Multiple Reaction Monitoring to Identify Sites of Protein Phosphorylation with High Sensitivity*

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Phosphorylation governs the activity of many proteins. Insight into molecular mechanisms in biology would be immensely improved by robust, sensitive methods for identifying precisely sites of phosphate addition. An approach to selective mapping of protein phosphorylation sites on a specific target protein of interest using LC-MS is described here. In this approach multiple reaction monitoring is used as an extremely sensitive MS survey scan for potential phosphopeptides from a known protein. This is automatically followed by peptide sequencing and subsequent location of the phosphorylation site; both of these steps occur in a single LC-MS run, providing greater efficiency of sample use. The method is capable of detecting and sequencing phosphopeptides at low femtomole levels with high selectivity. As proof of the value of this approach in an experimental setting, a key *Schizosaccharomyces pombe* cell cycle regulatory protein, Cyclin B, was purified, and associated proteins were identified. Phosphorylation sites on these proteins were located. The technique, which we have called multiple reaction monitoring-initiated detection and sequencing (MIDAS), is shown to be a highly sensitive approach to the determination of protein phosphorylation. ***Molecular & Cellular Proteomics 4:1134–1144, 2005.***

Protein phosphorylation is a key post-translational modification that governs biological processes (1). Its importance can be judged by the fact that ~2% of human genes encode protein kinases, and about 30% of proteins are phosphorylated (2). Systematic definition of these modifications in key proteins using sensitive methods will be of value in understanding the role of phosphorylation in normal and disease processes such as oncogenesis (3).

Currently the most sensitive techniques for protein analysis rely on MS (4). Various MS protocols have been developed to identify sites of phosphorylation. However, the definition of phosphorylation events remains technically challenging due to both the low occurrence of these peptides and their intrinsic physicochemical nature. This results in generally poor ionization and detection using standard MS techniques. These can be moderated using peptide derivatization (*e.g.* β-elimination and Michael addition) (5, 6). Derivatization using the same reactions offers specific protein cleavage dependent on phosphorylation (7). However, critical to the success of proteomic protocols is the simplicity and efficiency of these procedures prior to MS (8).

A further criterion for successful adaptation of phosphoproteomics protocols is sensitivity. Enrichment of phosphopeptides can be achieved via IMAC (9–11), graphite (12), and titanium oxide (13). These can be preceded by protein enrichment using phosphoamino acid-specific antibodies or phosphorylated consensus sequence-specific antibodies (14). Using highly enriched proteins current MS techniques can identify phosphorylation sites. These approaches include neutral loss scanning for the loss of a phosphate moiety (98 Da) from the parent ion (15) and precursor ion scanning either for an ion at *m/z* 79 in negative ion mode for Ser/Thr phosphorylation or an ion at *m/z* 216.043 for Tyr(P) in positive ion mode. Comparison of these techniques applied to gel-separated proteins yielded phosphorylation sites on peptides present at <200 fmol (16).

In cases where protein is highly abundant, precursor ion scan-based protocols have identified both novel and previously characterized phosphorylation sites (17–19). Notably these protocols have primarily been used for unfocused approaches where a large number of phosphoproteins and peptides have been identified in a complex mixture such as a whole cell lysate.

Where protein phosphorylation events on a specific target protein of interest are sought, however, the sensitivity of current MS techniques often requires overexpression of that protein to achieve quantities commensurate with precursor ion scanning methods. This is of concern to biologists as the protein is expressed at abnormal levels and often in an abnormal cell type. A more relevant method is direct purification from the cell type of interest. However, in a typical study, Zappacosta *et al.* (16) demonstrated a requirement for 200 fmol of protein (2×10^8 cells for a protein present at 100 copies/cell, assuming complete recovery of the protein during

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cell lysis, immunoprecipitation, gel separation, and in-gel digestion). This quantity of biological material for the purification of a specific protein target is not always available. Therefore, there is a growing requirement for objective, specific, and more sensitive protocols for discovery of multiple phosphorylation sites on individual proteins generated by standard, relatively rapid biochemical techniques. Previous work using hypothesis-driven analysis of a known protein developed by Chang *et al.* (20) demonstrated the power of this approach to detect the presence of phosphorylation. However, the approach they describe is unable to determine tyrosine phosphorylation or other post-translational modifications and often assumes that kinases only phosphorylate known consensus sequences.

A novel hybrid quadrupole linear ion trap mass spectrometer with functionalities of both triple quadrupole and linear ion trap instruments is now available (21, 22). This combination offers a unique opportunity for studies on post-translational modifications.

We describe an alternative, information-dependent method for the identification of sites of phosphorylation on target proteins. This technique is specifically designed to comprehensively analyze a single target protein rather than as a general phosphorylation screening tool. Because in most cases the biologist has a specific target protein of interest, the added sensitivity of this type of focused analysis is a great advantage. The first part of each analysis uses the instrument as a highly sensitive triple quadrupole mass spectrometer to perform multiple reaction monitoring (MRM)[1] analyses to screen for potential phosphopeptide signatures. The second phase of the experiment uses the third quadrupole as a linear ion trap for product ion detection to identify the peptide and the site of modification. This unique combination allows sensitive detection of phosphopeptides and identification of phosphorylation sites. We demonstrate below how this relatively simple approach can enhance the development of phosphoproteomics for the biologist.

EXPERIMENTAL PROCEDURES

Preparation of Samples—[Glu]Fibrinopeptide B (Sigma) was diluted using 2% (v/v) acetonitrile, 0.1% (v/v) formic acid and spiked at appropriate levels as a control compound. Bovine α-casein S1 and S2 (Sigma) was resuspended at a known concentration in 25 mM ammonium bicarbonate and digested by addition of modified porcine trypsin (Promega, Southampton, UK) at a 50:1 protein:enzyme ratio and incubated at 37 °C overnight. This provided a stock concentration of 20 pmol μl^{-1} that was further diluted and mixed using 2% (v/v) acetonitrile, 0.1% (v/v) formic acid to provide working standards. A single preparation of α-casein tryptic digested peptides was used throughout all experiments.

The six-protein mixture sample consisting of bovine serum albumin, alcohol dehydrogenase, apotransferrin, β-galactosidase, lysozyme, and cytochrome *c* was diluted from a 100 pmol lyophilized protein mixture digest (LC Packings, Amsterdam, The Netherlands) using 2% (v/v) acetonitrile, 0.1% (v/v) formic acid.

The *cdc13*$^+$ gene (23) was cloned into the NdeI site of pREP41PkC (29) and introduced into *cdc25.22 leu1.32* cells. Expression was induced by culturing at 25 °C for 31.5 h when the level of Cdc13-Pk equaled that of the native Cdc13 protein. The monoclonal antibody MAb336 was used to enrich for Cdc13 and associated proteins (24). Interphase (G_2 arrest) samples were generated by inducing cell cycle arrest through an increase in culture temperature from 25 to 36 °C. Cells were harvested 30 min after this culture had been returned to the permissive temperature of 25 °C.

Enriched fractions were separated by SDS-PAGE, and gels were stained with colloidal Coomassie Blue (www.lrf.umist.ac.uk). In-gel digests were performed as described previously (25).

Liquid Chromatography and Mass Spectrometry—For each experiment, 5 μl of sample were loaded onto a 15-cm $\times$ 75-μm inner diameter PepMap C_{18} 3-μm column (LC Packings) using a standard LC Packings UltiMate pump and FAMOS autosampler. Samples were desalted on line prior to separation using a microprecolumn (5-mm $\times$ 300-μm inner diameter) cartridge. The washing solvent was 0.1% formic acid delivered at a flow rate of 30 $\mu l\ min^{-1}$ for 3 min. Peptides were separated using a solvent gradient determined by the complexity of the sample mixture. Details of the gradients used for each sample is given in Supplemental Methods, Section 1.

Chromatography was performed on line to a 4000 Q-TRAP mass spectrometer (Applied Biosystems, Framingham, MA). All voltages and gas settings used are described in Supplemental Methods, Section 2.

Cdc2, Cyclin B, and Hsp60 were initially identified from ~10% of the digested sample by mass spectrometry using a QSTAR XL (Applied Biosystems) as described in Unwin *et al.* (26). Briefly the mass spectrometer was operated in information-dependent acquisition mode, which involves switching from MS to MS/MS mode on detection of doubly and triply charged species above a preset threshold. Data obtained are then combined and submitted to a protein database such as Swiss-Prot via Mascot (27) for protein identification.

MRM-initiated Detection and Sequencing (MIDAS)—For selective detection of phosphopeptides, a list of MRM transitions of potential phosphopeptides was generated either by manual calculation or by a software script developed by Applied Biosystems. In general, transitions were included for all tryptic peptides (maximum one missed cleavage) containing Ser, Thr, or Tyr residues with either one or two modifications and for doubly and triply charged species for the Q1 mass range 400–1600 *m/z*. The number of MRMs is dependent on various factors, including protein size, number of peptides following digestion, and the number of potential phosphorylation sites. It is, however, important to optimize the cycle time to around or below 10 s. Such a cycle time ensures that if the peak width is around 0.5 min it is highly probable that a peptide is scanned for and analyzed at least twice as it is eluted and that one of these analyses will occur at, or close to, the apex of its elution profile.

A large list of MRMs may be halved, and two separate analyses can be performed. Clearly other modifications can be included in the experiment such as oxidation of methionines or deamidation. Although these should be minimized during sample preparation, using a small proportion of sample in a standard identification experiment should quickly determine whether these modifications are present and whether they should be included in the list of MRM transitions.

The number of MRM transitions, dwell times, and cycle times used for each protein described in the study are provided in Supplemental Methods, Section 3. Collision energies (CE) for Ser(P) and Thr(P) peptides were calculated according to the equation $CE = (m/z \times 0.045) - 6.2$ for doubly charged peptides and $CE = (m/z \times 0.026) + 5.2$ for triply charged peptides. CE for Tyr(P) peptides is set at 60eV.

[1] The abbreviations used are: MRM, multiple reaction monitoring; MIDAS, MRM-initiated detection and sequencing; TIC, total ion chromatograph; XIC, extracted ion chromatograph; CE, collision energy; GluFib, [Glu]fibrinopeptide B.

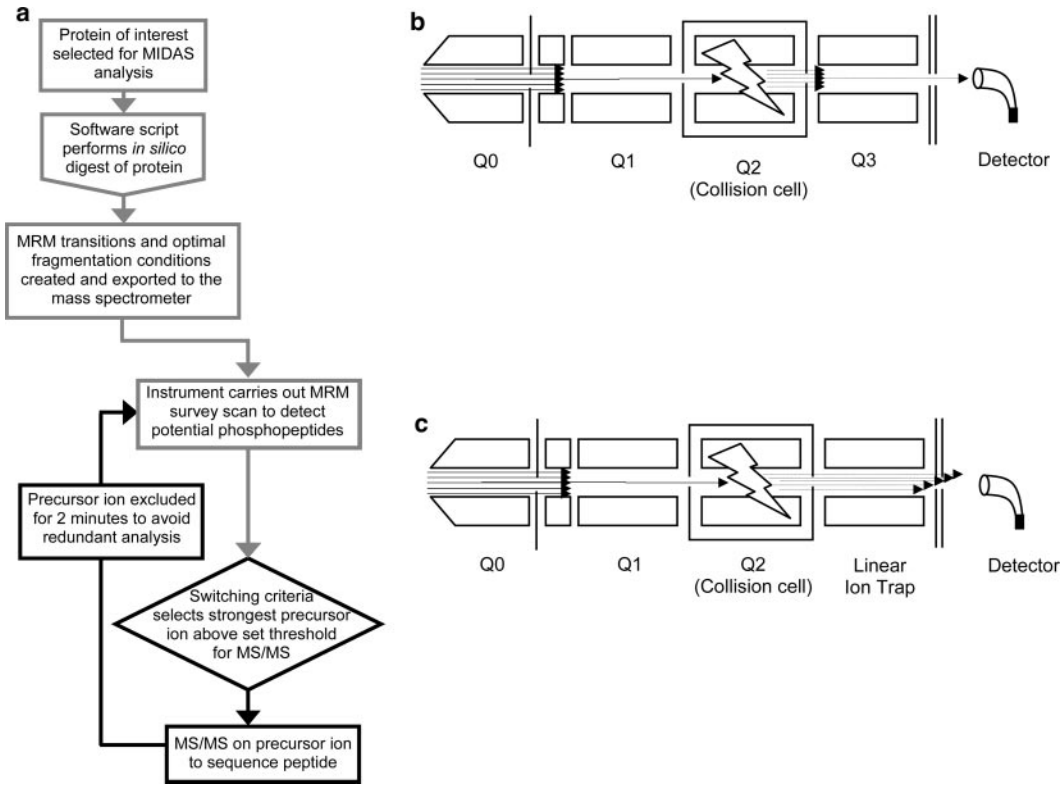


FIG. 1. **Description of the MIDAS technique.** *a*, flow diagram of the MIDAS protocol showing the main stages of analysis. Once the protein of interest has been identified, MRM Builder software generates a set of precursor-to-product ion transitions for potential phosphopeptides along with appropriate collision energies (*light outline*). The mass spectrometer uses this data to perform sequential scan cycles and selects suitable peptides for MS/MS analysis. These peptides are then excluded from further study for a period of 2 min (*heavy outline*). *b*, schematic representation of the 4000 QTRAP with Q3 configured to operate as a quadrupole mass filter. During this MRM phase of the acquisition cycle, precursor ions are sequentially and selectively transmitted through Q1 for a defined dwell time. In the collision cell (Q2) the ion is fragmented via CID, and the appropriate MRM product ion is selectively transmitted through Q3 to the detector. *c*, schematic representation of the 4000 QTRAP with Q3 configured to operate as a linear ion trap. During this MS/MS phase of the acquisition cycle, upon CID fragmentation of the precursor ion all product ions are allowed into the linear ion trap (Q3) where they are accumulated for a set fill time. All product ions are then mass selectively ejected from the trap generating a product ion spectrum at the detector.

The mass spectrometer was instructed to switch from MRM to enhanced product ion scanning mode when an individual MRM signal exceeded 100 counts. Each precursor was fragmented a maximum of twice before being excluded for 2 min. Enhanced product ion scanning was performed using parameters detailed in Supplemental Methods, Section 2. Data were initially analyzed by submitting the MS/MS data to Mascot with a peptide tolerance setting of ±0.02 Da. This proves successful as the exact parent mass is known and defined in the MRM transition. Hence the search can be performed using the actual mass of the peptide of interest rather than a less accurate mass generated by the instrument. As such, few false positive database "hits" are generated. All Mascot searches were performed against a species-specific NCBInr database with trypsin plus one missed cleavage, phosphorylation as a variable modification, and an MS/MS tolerance of 0.5 Da. In addition, data were examined manually using the Applied Biosystems BioExplore feature of the Analyst 1.4 software package.

RESULTS

Multiple Reaction Monitoring-based Phosphorylation Site Analysis—This method uses MRM as a selective trigger for enhanced product ion (MS/MS) scans on phosphopeptides. MRM is a highly sensitive function of triple quadrupole mass spectrometers where the first quadrupole transmits only an ion of specific *m/z* (Q1 mass). This ion is then fragmented in the second quadrupole. The third quadrupole is set to transmit a specific product ion (Q3 mass). Detection of this product is therefore diagnostic with multiple "Q1 to Q3 transitions" assayed in a single experiment. The time taken to analyze each transition is termed the "dwell time." MRM requires two ions to generate a positive result, making it very specific with very low background, enhancing sensitivity of detection.

We exploited these features to scan for phosphopeptides and termed this approach MIDAS. The overall scheme of the method is outlined in Fig. 1*a*. Initially a candidate list of MRM transitions is generated. The masses of theoretical phosphopeptides are calculated by addition of 80 to the peptide mass for every "phosphorylatable" residue present. The Q1 mass is calculated for this peptide in doubly/triply charged form with up to two phosphate additions. The Q3 mass is calculated as either loss of 98 Da for each putative Ser(P) or Thr(P) or generation of a 216.0 ion for tyrosine-containing peptides. An example is shown in Table I. A dwell time and collision energy are then added.

TABLE I
MRM transition masses along with optimal collision energies calculated for the Cdc2 tryptic peptide IGEGTYGVVYK with two potential modifications and a charge state of +2 or +3 selected

Q1 mass	Q3 mass	Amino acid sequence	Fragment type	Fragment mass	Charge	Collision energy
				Da		*eV*
673.2	624.2	IGEGpTpYGVVYK	Loss of	98.0	+2	24.1
673.2	216.0	IGEGTpYGVVpYK	Fragment	216.0	+2	60.0
633.2	584.2	IGEGpTYGVVYK	Loss of	98.0	+2	22.3
633.2	216.0	IGEGTpYGVVYK	Fragment	216.0	+2	60.0
449.1	416.5	IGEGpTpYGVVYK	Loss of	98.0	+3	16.8
449.1	216.0	IGEGTpYGVVpYK	Fragment	216.0	+3	60.0
422.5	389.8	IGEGpTYGVVYK	Loss of	98.0	+3	16.1
422.5	216.0	IGEGTpYGVVYK	Fragment	216.0	+3	60.0

An MRM analysis based on this list is then performed (Fig. 1*b*). After each cycle, the strongest signal above a set threshold is selected, and the instrument switches mode to perform the MS/MS analysis (Fig. 1*c*) on the relevant precursor ion whose *m/z* is inferred from the Q1 mass in the MRM. This generates sequence data, confirming the peptide identity, and possibly the site(s) of phosphorylation.

Assessment of MIDAS Sensitivity Using Standard Proteins—The sensitivity and selectivity of this method were assessed using a model phosphoprotein, α-casein. α-Casein was selected to allow comparison with existing phosphorylation analysis methods (19, 28) where limits of detection are published.

A MIDAS experiment comprising 65 MRMs to identify α-casein S1 phosphopeptides is presented in Fig. 2. 10 fmol of α-casein spiked with 4 fmol of [Glu]fibrinopeptide B (GluFib), used as a positive control, were loaded onto the column. The addition of GluFib was important at this stage as a control when dilution of α-casein below the limit of detection was performed.

Fig. 2*a* shows the total ion current (TIC) for this experiment. Because of the selectivity of the MRM survey scan, the ion current remains low until the instrument "switches" to perform a product ion scan. Fig. 2*b* shows the TIC for just the MRM experiments. A low background was recorded except where the GluFib and phosphopeptides were eluted. Fig. 2, *c* and *d*, shows the extracted ion current (XIC) for two selected transitions. A single peak was obtained, implying that the instrument switched on a peptide rather than a nonspecific background ion. Fig. 2, *e* and *f*, shows the MS/MS product ion spectra for GluFib and the α-casein S1 protein phosphopeptide VPQLEIVPNpSAEER (where pS is phosphoserine), respectively. The y ion coverage enabled unambiguous identification of the (phospho)peptide.

Using MIDAS, the peptide sequence and number of phosphorylation sites are inferred from the MRM transition trigger. Thus, location of phosphorylation site(s) may require few fragment ions in certain cases. Because the sensitivity of MRM is much higher than for product ion scanning, the sensitivity of phosphopeptide and phosphorylation site detection is improved. Fig. 3, which depicts data from MIDAS analysis of either α-casein S1 or S2, illustrates this point. Fig. 3, *a* and *b*, shows product ion spectra that define phosphorylation sites on α-casein S1 and confirm phosphorylation of an α-casein S2 peptide when loaded onto the column at 10 fmol of α-casein (a mixture of S1 and S2). Even when the column loading was reduced to 5 fmol, the α-casein S1 (trypsin) missed cleavage phosphopeptide YKVPQLEIVPNpSAEER is identified (Fig. 3*c*). Fewer y ions were generated, but there remains sufficient information to confirm the site of phosphorylation. For the α-casein S2 peptide (Fig. 3*b*), the spectrum confirms peptide identity. Phosphorylation was implied from the precursor mass and positive MRM, and the spectrum shows modification on either Ser-6 or Thr-7. A more focused MIDAS analysis to detect only this peptide with a longer acquisition of product ion spectra demonstrates phosphorylation on Ser-6 (see Supplemental Fig. 1) showing the utility of careful MRM experiment design. Two peptides were detectable that contain the *same* phosphorylated serine due to inefficient tryptic cleavage. Therefore we can characterize phosphopeptides in the low femtomole region, with and without missed cleavage, by MIDAS.

Assessment of MIDAS Sensitivity for Proteins in a Mixture—Often it is not possible to obtain completely pure samples of a protein. Further experiments were performed to assess whether MIDAS can identify phosphorylation sites on specific proteins in a simple mixture such as that obtained from a conventional one-dimensional gel band of an immunoprecipitate. To model this situation, a mixture of six non-phosphorylated proteins "spiked" with α-casein was used. Fig. 4*a* shows the TIC from a standard protein identification experiment on 500 fmol of each non-phosphorylated background protein with 20 fmol of α-casein. The *inset table* shows a Mascot (24) database search illustrating that, although we identified all proteins present at 500 fmol, α-casein S1 and S2 are not detected (due to the high level of other peptide ions). Fig. 4, *b* and *c*, shows that when MIDAS was performed for α-casein S1, two potential phosphopeptides produce positive MRMs, leading to high quality MS/MS data (Fig. 4, *d* and *e*) confirming the presence of phosphorylation on these peptides.

Identification of Phosphorylation Sites on Cell Cycle Regulatory Proteins—We next demonstrated application of MIDAS by identifying phosphorylation sites on a number of yeast

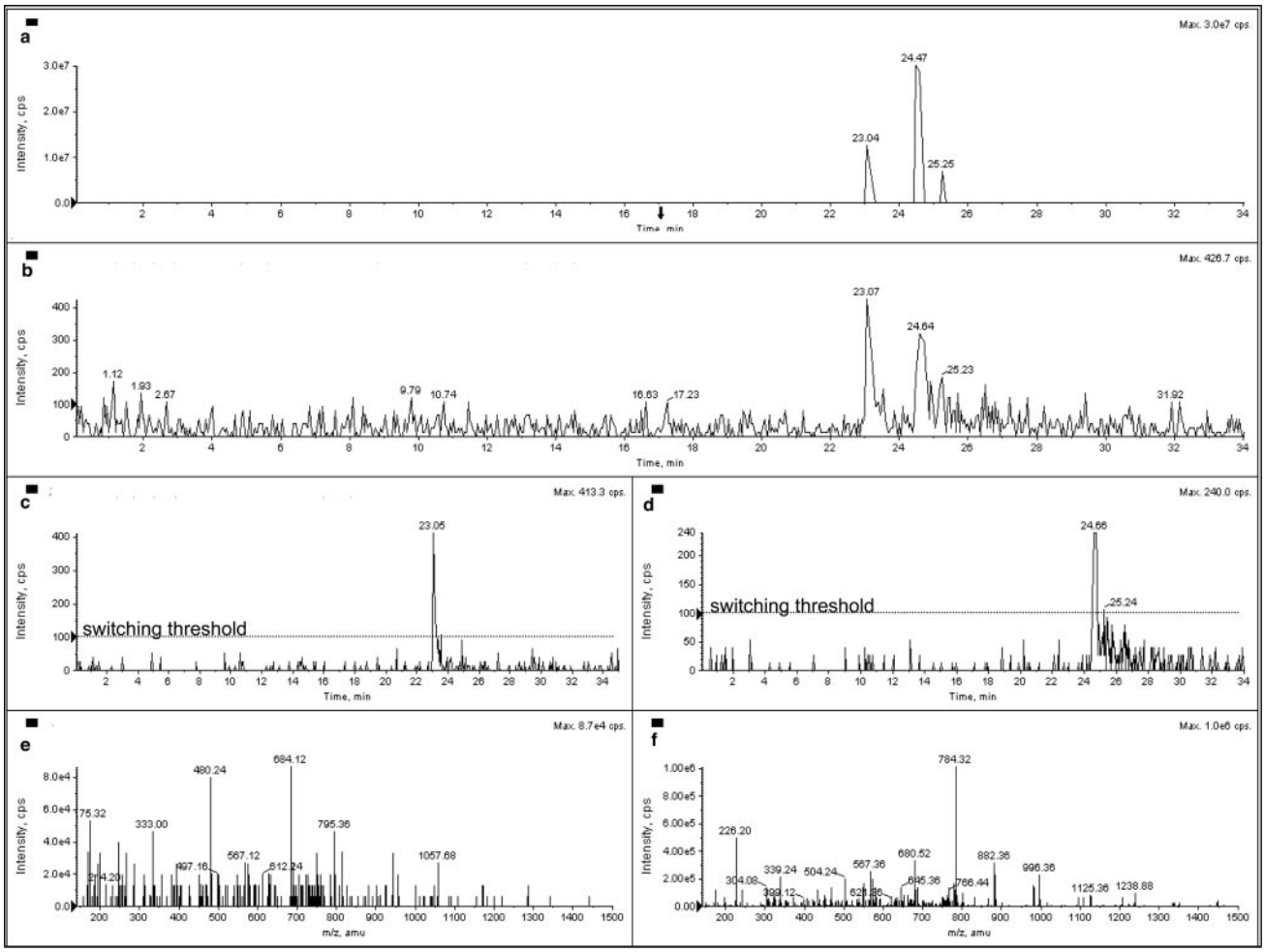


FIG. 2. **MIDAS analysis of a mixture of GluFib and α-casein (LC column loadings of 4 and 10 fmol, respectively).** *a*, TIC for the MIDAS experiment clearly showing points in the analysis where product ion scans were triggered by MRMs that exceeded the preset threshold. *b*, TIC for the MRM phase of the experiment showing a total background signal of around 100 counts per scan (*cps*). Peaks at 23.07 and 24.64 min correspond to the elution of GluFib and an α-casein phosphopeptide. *c*, XIC for GluFib transition 785.8 $>$ 684.3 showing a low background signal for this transition and elution of the peptide at 23.05 min. As the peptide elutes from the column, the MRM signal exceeds the switching threshold of 100 counts per scan, triggering a product ion scan. *d*, XIC of the 830.9 $>$ 781.9 transition showing a low background signal for this transition and elution of phosphopeptide at 24.66 min. As the peptide elutes from the column, the MRM signal exceeds the switching threshold of 100 counts per scan, triggering a product ion scan. *e*, MS/MS analysis triggered by a positive signal from the 785.8 $>$ 684.3 transition confirming the presence of GluFib. *f*, MS/MS analysis triggered by a positive signal from the 830.9 $>$ 781.9 transition confirming the presence of an α-casein phosphopeptide and its site of phosphorylation.

proteins. Cyclin B binds Cdc2 to form the mitosis protein factor that controls commitment to mitosis. As the two proteins associate, Cdc2 is phosphorylated at threonine 167 to activate the complex. As the complex forms prior to mitosis, phosphorylation on a second site, tyrosine 15, inhibits the complex until entry into M phase whereupon tyrosine 15 is dephosphorylated (29, 30). Co-immunoprecipitation of this complex from fission yeast in either interphase or M phase yielded a series of proteins detected by one-dimensional SDS-PAGE and colloidal Coomassie Blue staining (Fig. 5*a*). Several bands were excised and digested, and their identities were confirmed by standard methods using the QSTAR XL. Cyclin B, Cdc2, and Hsp60 (Mascot scores 695, 733, and 59, respectively) were selected as paradigm proteins. MIDAS analysis of Cyclin B from the mitotic sample successfully identified a novel site of phosphorylation on serine 111 (SIIPATDDEPApSK, Fig. 5*b*), a predicted target sequence of protein kinase C. It is noteworthy that a recent analysis of Cyclin B phosphorylation argued that it was extremely unlikely such novel sites existed (31), thus the value of MIDAS is proven within this biological context. Analysis of Cdc2 isolated from cells in interphase identified phosphorylation at the key sites on the activating threonine (Thr-167; NYpTHEIVTLWYR (where pT is phosphothreonine); Fig. 5*c*) and the inhibitory tyrosine (Tyr-15; IGEGTpYGVVYK (where pY is phosphotyrosine); Fig. 5*d*). Using standard controls, we estimated that this gel band contained ~350 fmol of protein of which 10% was used to confirm protein identification and 25% (87.5 fmol)

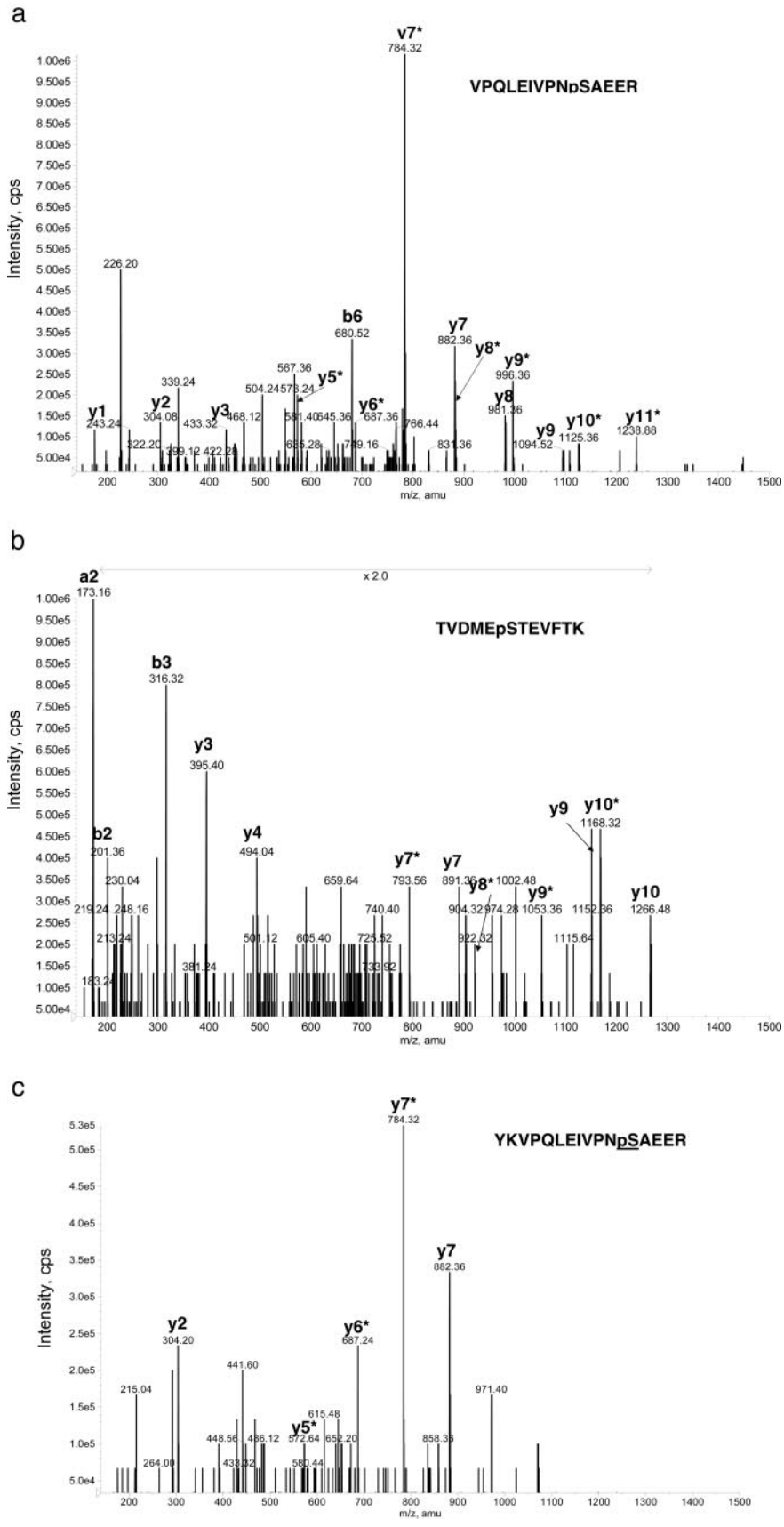


FIG. 3. **Evaluation of method sensitivity using the model protein α-casein.** *a*, MS/MS spectrum of the α-casein S1-derived phosphopeptide VPQLEIVPNpSAEER obtained from a 10-fmol sample of α-casein S1 and S2. *b*, MS/MS spectrum of the α-casein S2-derived phosphopeptide TVDMEpSTEVFTK obtained from a 10-fmol sample of α-casein S1 and S2. The site of phosphorylation was confirmed at 20 fmol (data not shown). *c*, MS/MS spectrum of the α-casein S1-derived phosphopeptide YKVPQLEIVPNpSAEER obtained from a 5-fmol sample of α-casein S1 and S2. In all cases an *asterisk* denotes a dehydroalanine-containing ion. *cps*, counts per scan.

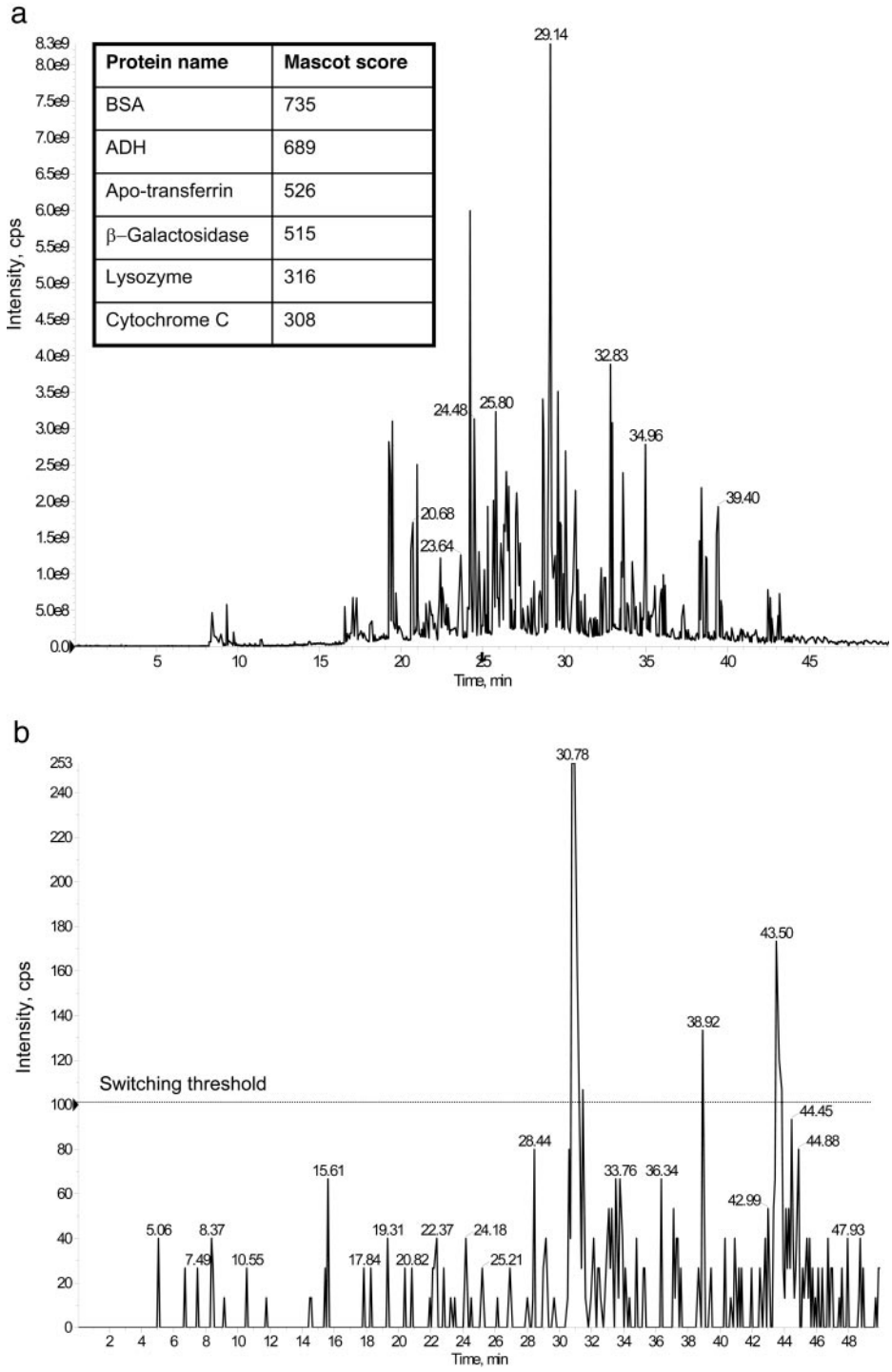


FIG. 4. **Analysis of 20 fmol of α-casein S1 and S2 tryptic digest in the presence of a six-protein mixture.** *a*, TIC of 500 fmol of a six-protein mixture containing 20 fmol of α-casein. The *inset* shows the proteins identified using a standard LC-MS/MS protocol along with the Mascot score. *b*, XIC of the 830.9 > 781.9 transition showing the presence of phosphopeptide at 30.78 min. *c*, XIC of the 651.3 > 618.7 transition showing the presence of phosphopeptide at 30.34 min. *d*, MS/MS spectrum of the peptide *m/z* 830.90 confirming a phosphorylation in the α-casein S1 peptide VPQLEIVPNpSAEER. *e*, MS/MS spectrum of the peptide *m/z* 651.32 confirming a phosphorylation in the α-casein S1 peptide YKVPQLEIVPNpSAEER, a missed trypsin cleavage peptide containing the same phosphorylation site. In all cases an *asterisk* denotes a dehydroalanine-containing ion. *cps*, counts per scan; *ADH*, alcohol dehydrogenase.

was sufficient to enable identification of phosphorylation sites. These sites were also identified in the mitotic sample (data not shown). A novel serine phosphorylation site was also identified from Hsp60 (Ser-77; NVLIDQPFGpSPK; Supplemental Fig. 2).

DISCUSSION

The value of MIDAS lies in the sensitivity and selectivity afforded by the MRM analysis. Because the instrument only allows ions of a single characteristic *m/z* into the collision cell and ions of a second diagnostic *m/z* from the collision cell to the detector, the background signal becomes exceedingly low. This enhanced signal-to-noise ratio allows more sensitive detection of phosphopeptides. As shown in Fig. 3*c*, the generation of quality product ion spectra, rather than detection of the phosphorylated species, is the limiting factor in terms of the sensitivity of this approach. Even at these low levels, the

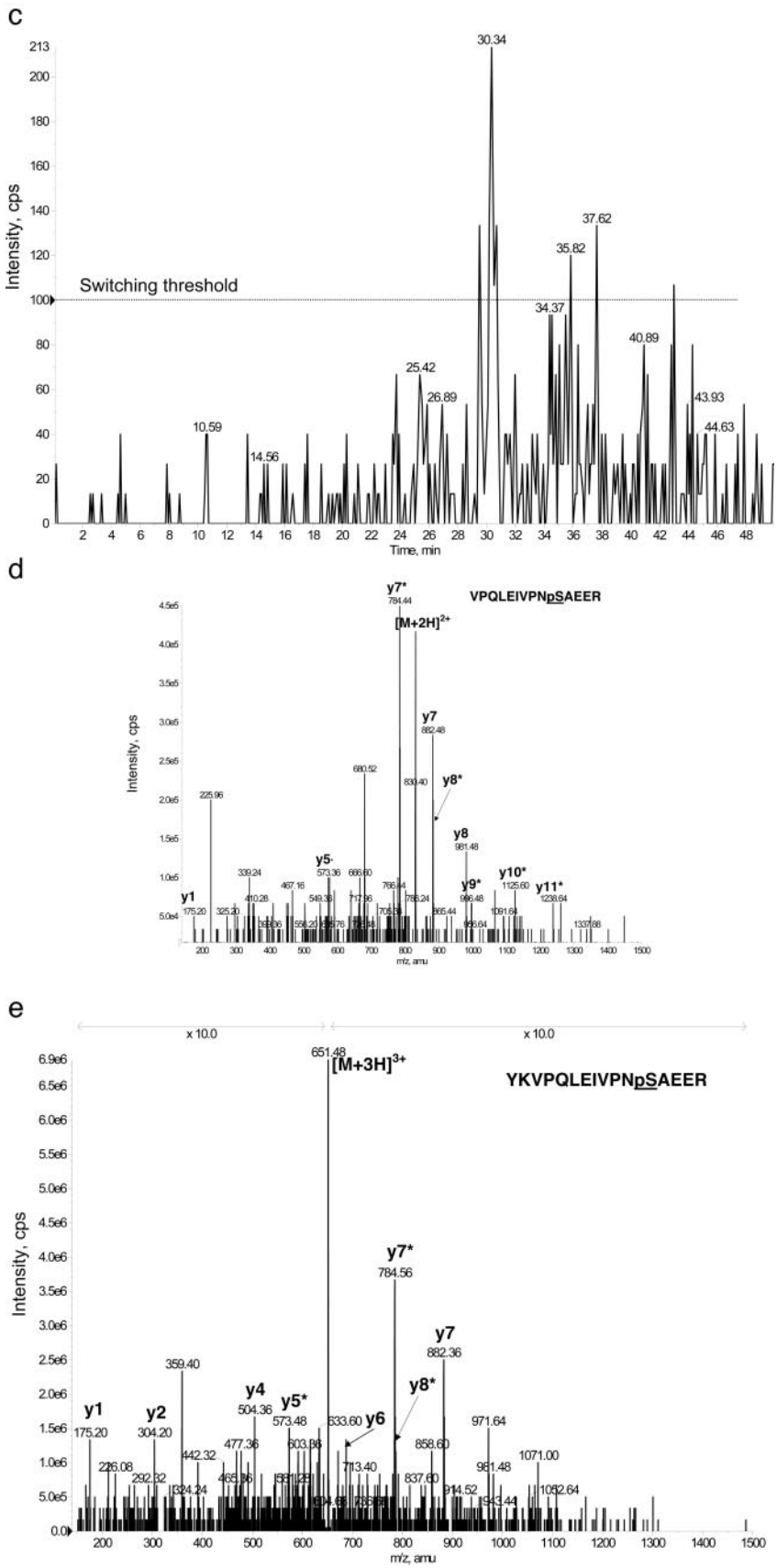
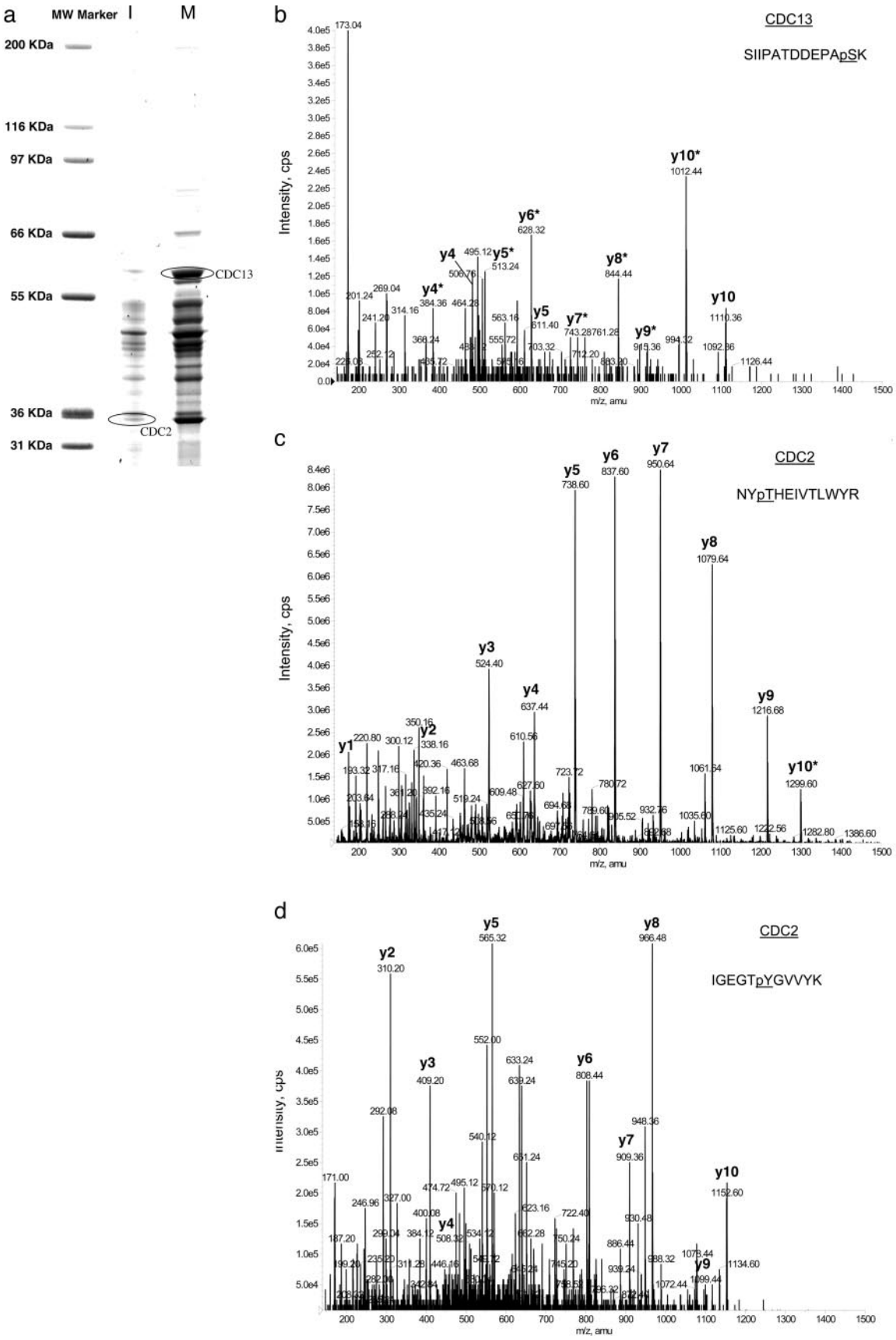


FIG. 4—*continued*



phosphopeptide was recognized as such by the MRM, and these data can be used to infer phosphorylation on this peptide even if it does not necessarily confirm the specific amino acid on which the modification occurs.

In theory and in practice our protocol generated few false positives. Compared with established phosphate scanning methods, definition of a precursor mass equivalent to that of a phosphopeptide vastly reduces false positive results due to generation of b2 ions (216 Da) interfering in a phosphotyrosine peptide analysis. In the cases of phosphoserine and phosphothreonine neutral loss analysis, false positives resulting from generation of a peptide fragment of "diagnostic" *m/z* or loss of valine (99 Da) are diminished. In addition, because the accurate precursor mass is known, database searching can be performed with very tight tolerances. This specificity is especially important if the protein is present in a mixture.

Even with 20 fmol of phosphoprotein in a mixture of other proteins at 500 fmol (Fig. 4), no false positives were generated. This sample, prepared specifically to test the specificity of the MRM scanning approach, also allowed direct comparison to published methods (28). Because this 20 fmol is a mixture of two α-casein proteins and the phosphopeptides found in this experiment are a missed cleavage around the same phosphorylation site, we are effectively detecting peptides present at significantly less than the 20 fmol of total protein loaded onto the column. The sensitivity of this method represents a significant improvement of approximately an order of magnitude compared with analysis of a similar sample on this instrument using precursor ion scanning, which detected α-casein phosphopeptides in a protein mixture at 75 fmol (28). This type of sample represents an extreme "worst case" scenario where the biologist has very high levels of contaminating protein, yet MIDAS is still capable of identifying phosphorylation sites on the target protein. MRM analysis therefore provides a more sensitive and specific scanning method for phosphopeptides from a known target protein than existing methodologies.

We confirmed the effectiveness of the method for biochemical samples prepared in a standard format. Phosphorylation sites on proteins from *Schizosaccharomyces pombe* isolated by immunoprecipitation and one-dimensional gel electrophoresis were taken for successful identification of a novel site of serine phosphorylation on mitotic Cyclin B (argued to be a completely phosphor-mapped protein) and two previously observed phosphorylation sites in a Cyclin B-interacting protein, Cdc2. In addition, a novel serine phosphorylation site was identified on Hsp60. These data demonstrate that MIDAS can be used to obtain information regarding novel phosphorylation sites using quantities of protein that are feasibly obtained from real biological model systems.

Creating the MRM experiment peptide list is relatively simple and can be tailored to search for specific predicted phosphorylation sites, at even greater sensitivity, by increasing the dwell time or the number of MS/MS scans performed on a specific peptide precursor. For a protein that generates a large number of MRMs, the experiment is easily fractionated keeping the cycle time below 10 s. Because we did not use the entire sample for the identification of phosphopeptides in our yeast protein samples, this is a real solution to this problem. It should be mentioned, however, that an experiment comprising 172 MRMs was performed and successfully identified sites of Cyclin B phosphorylation. It is important to note that for the samples analyzed here not all potential phosphorylation sites and charge states were considered; rather a degree of intelligent filtering was used to limit the number of MRMs to a reasonable level. The reasoning behind this filtering is described in the supplemental methods for each protein. For a complete screen of multiple sites and charge states the MRM transition list often becomes large and requires splitting between multiple injections. For example, Cdc2 would require 374 MRM transitions for both +2 and +3 charge states with Ser/Thr/Tyr being potentially phosphorylated (maximum of three per peptide), assuming one missed cleavage and possible methionine oxidation. This would require four injections of the sample. If zero missed cleavages are considered this is reduced to one injection of 124 MRMs. The level of missed cleavages and methionine oxidation can be assessed in a standard protein identification experiment on the sample and can therefore be included or excluded from the MRM analysis as required.

Clearly MIDAS is not a panacea. The intrinsic biochemical nature of phosphopeptides that causes their adherence to surfaces during LC-MS/MS or their poor ionization is not addressed in this study. However, further experiments such as phosphopeptide enrichment by IMAC or strategies to improve ionization efficiency of phosphopeptides will increase the sensitivity of this approach further. Use of other proteases will also improve protein coverage and may also improve ionization of peptides containing phosphorylated residues. Where sufficient sample is available, several techniques should be combined to fully characterize all sites of phosphorylation on a protein of interest. However, given the improvement in sensitivity and selectivity that MIDAS offers over other LC-MS/MS-based methodologies, it is clear that this method is successful where small amounts of protein are available.

FIG. 5. **Analysis of Cyclin B-interacting proteins using the MIDAS technique.** *a*, one-dimensional SDS-PAGE image of Cyclin B-interacting proteins in both interphase (*I*) and M phase (*M*) stained with colloidal Coomassie Blue. Paradigm proteins Cyclin B (Cdc13) and Cdc2 are highlighted. *b*, MIDAS-derived MS/MS spectrum of the Cyclin B phosphopeptide SIIPATDDEPApSK during M phase. An *asterisk* denotes a dehydroalanine-containing ion. *c*, MIDAS-derived MS/MS spectrum of the Cdc2 phosphopeptide NYpTHEIVTLWYR. An *asterisk* denotes a dehydroamino-2-butyric acid-containing ion. *d*, Cdc2 phosphopeptide IGEGTpYGVVYK during interphase. *MW*, molecular weight; *cps*, counts per second.

None of the *S. pombe* proteins analyzed were overexpressed, making MIDAS a highly valuable tool in rapidly assessing phosphorylation in a target protein of interest without the need for lengthy cloning, overexpression, and purification techniques. Furthermore MIDAS is also applicable to the analysis of other key post-translational modifications such as acetylation (results not shown). Given the semiquantitative nature of MRM, MIDAS can also theoretically be extended to perform relative quantitation of modified peptides in a protein from different samples without the need for radioactivity, stable isotopes, or the production of modification-specific antibodies. Therefore, MIDAS provides a sensitive and flexible approach to protein post-translational modification identification and analysis.

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