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ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2008

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Characterization of the Human Cerebrospinal Fluid Phosphoproteome by Titanium Dioxide Affinity Chromatography and Mass Spectrometry

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Biomarkers in the cerebrospinal fluid (CSF) may be important for the diagnosis of chronic degenerative disorders in the central nervous system including dementia. Existing CSF biomarkers for dementia, however, are relatively nonspecific. More specific markers may be found by targeting investigations based on knowledge of the molecular pathology of the disease in question. In Alzheimer's disease, hyperphosphorylation of the tau protein is a characteristic feature and thus a comprehensive characterization of the phosphoproteome of the CSF may be pursued to obtain a complete picture of phosphorylation aberrations in health and disease. Toward that goal we here describe a method for a comprehensive isolation and identification of phosphorylated tryptic peptides derived from CSF proteins using a simple sample preparation step and titanium dioxide-affinity chromatography followed by MALDI-TOF or LC-MS/MS linear ion-trap-FT mass spectrometry. Whereas not all previously reported phosphoproteins were found in normal CSF, we detected 56 putative novel phosphorylation sites in 38 proteins in addition to known sites. The approach seems to be a promising foundation for the discovery of new biomarkers embedded in the CSF phosphoproteome.

Cerebrospinal fluid (CSF) surrounds the entire central nervous system (CNS) and contains a mixture of proteins that is distinctly different from the profile of proteins in other body fluids like blood. The analysis of CSF proteins is of clinical biochemical importance because of the proximity to the brain and spinal cord allowing changes in CNS proteins and peptides to be reflected in the CSF proteome. Thus, CSF is suitable for studying disease specific activities such as e.g. the levels of tau protein and amyloid-β 1−42 peptide in the diagnosis of Alzheimer's disease (AD)[1] and Creutzfeldt-Jakob disease[2] and the presence of oligoclonal bands, reflecting CNS-localized immunoglobulin synthesis, in the diagnosis of multiple sclerosis.[3]

Although these biomarkers are of some use, more accurate and sensitive biomarkers are severely needed in the field of chronic degenerative neurological disorders. One possible way to accelerate the discovery of novel biomarkers in biological fluids is to use multianalyte approaches such as proteomic methods including two-dimensional gel electrophoresis and mass spectrometry (MS). MS has been widely used to characterize the proteomes of various biofluids during the past decade, and MS-based proteomic studies of CSF have also been carried out by different groups during recent years.[4–9] One hurdle in MS-based proteomics of biofluids is to be able to identify proteins present in high concentrations at the same time as identifying proteins or protein modifications that may be present in much lower concentrations. This issue is partly alleviated when focusing analysis on subfractions chosen by considering the context, e.g. the apparent association of hyperphosphorylation with AD.[10] In other prefractionation techniques, immunological tools or exploitation of physicochemical characteristics of the analytes may be used to reduce the input complexity.[11–16]

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10.1021/ac800835y CCC: $40.75
Published on Web 08/15/2008

Overall, prefractionation approaches where the separation is specific to post-translational modifications (PTMs) are highly warranted. Protein PTMs such as phosphorylation, glycosylation, methylation, and many others[17,18] play a significant role in transcriptional and translational regulation, metabolism, degradation, cellular signaling, and structural integrity of proteins. Phosphorylation is recognized as one of the major PTMs. Accordingly, several methods have been applied to visualize, enrich, and identify phosphorylated proteins and peptides (for review see refs 19, 20). Enrichment of phosphoproteins has been performed using immunoprecipitation with phosphoamino acid-specific antibodies. A limitation of the immunoprecipitation and the covalent tagging methods is that they only target phosphotyrosine containing proteins or phosphothreonine/phosphoserine proteins and not both types. Covalent modification and tagging of phospho-groups through β-elimination[16] or transient carbodiimide formation[21] has also been applied. Immobilized metal affinity chromatography with Fe^{3+} or Ga^{3+} has been used successfully to enrich for phosphorylated peptides as well,[14,15] although this method is of limited specificity and also captures peptides with accumulations of negatively charged amino acids.

Recently, a novel affinity chromatographic method based on titanium dioxide (TiO_2) has been developed to enrich for phosphopeptides[22] and sialylated glycopeptides[23] in complex mixtures. The increased specificity of this method makes it possible to study low abundant phosphopeptides in biofluids.

Here, we use optimized sample preparation and phosphopeptide enrichment using TiO_2 chromatography to obtain a baseline map of the phosphoproteome in CSF from control individuals. The approach is based on CSF-preconcentration, a two-stage digestion scheme, deglycosylation, TiO_2 enrichment, and advanced mass spectrometric analysis. In total, we found 56 novel phosphorylation sites in 38 proteins. These results show, beside confirmation of known phosphorylation sites, the existence of a large number of previously unknown phosphorylation sites in phosphoproteins in CSF as well as the discovery of novel phosphorylated isoforms of proteins hitherto not known to be phosphorylated.

MATERIALS AND METHODS

Materials. Titanium dioxide beads were from GL Science (Titansphere, 5 μm). GELoader tips were from Eppendorf (Hamburg, Germany). Trifluoroacetic acid and glycolic acid were from Fluka (Sigma, St. Louis, MO), and 2.5-dihydroxybenzoic acid (DHB) was obtained from Aldrich (Sigma, St. Louis, MO). Acetonitrile was from Sigma (St. Louis, MO). Modified trypsin was from Promega (Madison, WI). Endoglycosidase F (PNGaseF) was from Roche Diagnostics (Manheim, Germany). Poros Oligo R3 reversed-phase chromatography resin was from Applied Biosystems (Framingham, MA). Orthophosphoric acid (85%, v/v) was from J.T. Baker. Ammonium solution (25%) and formic acid was from Merck & Co., Inc. (Rahway, NJ); 3 M Empore C8 discs were from 3 M Bioanalytical Technologies (St. Paul, MN). All reagents in the experiments were analytical grade or better, and the water from a Milli-Q system (Millipore, Bedford, MA).

Lumbar CSF Samples. The patient group consisted of nondemented patients with a suspected neurological disorder. The patients underwent lumbar puncture and the CSF was sent to the Department of Clinical Biochemistry and Immunology at Statens Serum Institut for oligoclonal band analysis. The CSF was collected in polypropylene vials and stored at −20 °C. The CSF samples used here were from 25 male and female subjects between 20 and 70 years of age and were devoid of oligoclonal bands and raised IgG levels. Approximately 1 mL of CSF from each patient sample was taken out, pooled, aliquoted, and stored at −80 °C until it was used. The total protein concentration in the CSF pool was 500 μg/mL.

Protein Concentration and Proteolytic Cleavage of Proteins from CSF. Protein concentration was measured using QuantIT (Invitrogen). Control CSF was concentrated 70 x by volume using 3 kDa cutoff spin columns (Microcon Ultracel YM-3, Millipore) to a total protein concentration of approximately 35 mg/mL and subsequently diluted 1:1 in 8 M urea/0.4 M NH_4HCO_3. For reduction, dithiothreitol (Sigma) was added to a final concentration of 10 mM and the mixture was incubated at 56 °C for 45 min. For alkylation, the mixture was cooled to room temperature and iodoacetamide was added to a final concentration of 20 mM and incubated at room temperature for 45 min in the dark. Lys-C endoproteinase (Wako Chemicals, USA) was added to the solution (1:50 w/w) and the mixture was incubated at 37 °C for 3 h. The solution was diluted 4× with water and trypsin (Promega) was added (1:50 w/w). The digestion mixture was incubated at 37 °C overnight.

Enrichment of Phosphopeptides using TiO_2. The phosphopeptides were enriched as previously described.[22,24] An approximately 200 μg sample was prepared for TiO_2-affinity chromatography. Briefly, 0.5 U of PNGase F was added to the Lys-C and trypsin digested sample and incubated for 3 h at 37 °C prior to the phosphopeptide enrichment to remove N-linked sialic-acid containing glycan structures (Figure 1). The removal of the N-linked sialic-acid containing glycopeptides is performed to avoid having a reduction in column capacity and specificity as sialic acid-containing glycans will bind to TiO_2.[23] The digested and PNGase F-treated CSF was diluted 1:5 in TiO_2 loading buffer (1 M glycolic acid, 5% trifluoroacetic acid (TFA), 80% acetonitrile) and loaded on TiO_2 microcolumns of approx 4−5 mm in length as previously described.[22] The columns were washed with 20 μL loading buffer and 30 μL washing buffer (80% acetonitrile, 1% TFA). The phosphopeptides were eluted with 40 μL NH_4OH, pH 11 and the eluate was acidified with 4 μL 100% formic acid.

The acidified phosphopeptides were desalted on R3 microcolumns as previously described.[22] The sample was either eluted directly onto the MALDI-target (see below) or eluted with 50% acetonitrile, 0.1% TFA, lyophilized, and redissolved in 10 μL 0.1% formic acid for LC-MS analysis.

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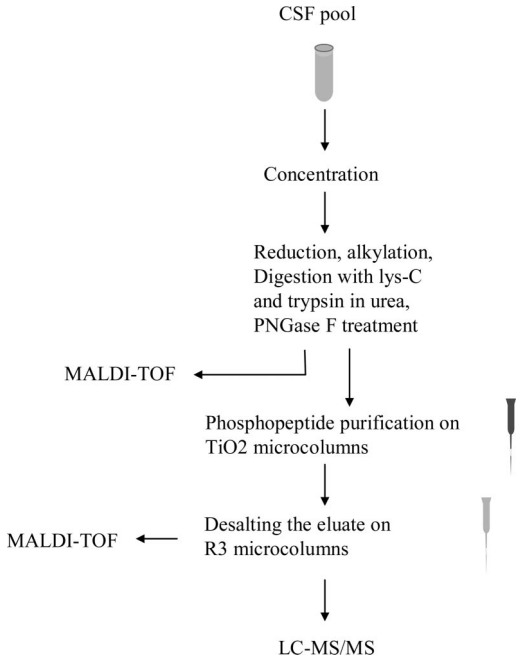

Figure 1. Flow-diagram showing the experimental setup. PNGase F:Endoglycosidase F.

MALDI-TOF Mass Spectrometry. MALDI-TOF was performed on a Bruker Ultraflex II MALDI-TOF-TOF (Bruker Daltonics). The purified phosphopeptides were eluted from the R3 microcolumn directly onto the MALDI-target using DHB matrix (20 mg/mL DHB in 70% acetonitrile, 1% orthophosphoric acid and 0.1% TFA). All spectra were obtained in positive ion mode. The spectra were processed using the MoverZ software (http://bioinformatics.genomicsolutions.com/MoverZNt.html).

LC-MS/MS. The redissolved sample was concentrated and desalted on an in-house packed fused silica 1.5 cm precolumn (100 μm inner diameter, 375 μm outer diameter, ReproSil, C18 AQ 3 μm (Dr. Maisch, Germany)) and eluted at 250 nL/min by an increasing concentration of acetonitrile. The peptides were eluted onto an in-house packed 8 cm fused silica C18 analytical column (Reprosil, Dr. Maisch, Germany) (50 μm, I.D., 375 μm, O.D.) using an EASY nLC system (Proxeon A/S, Odense, Denmark) with a 65 min gradient from 0−50% B buffer (A buffer: 0.1% formic acid, B buffer 0.1% Formic acid/90% acetonitrile) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The peptides were detected in the Orbitrap based on an intensity of 1000 counts. A total of 5 of the most intense peptides were selected for each MS scan for fragmentation using multistage activation. The fragmentation of phosphopeptides was performed using multistage activation in the linear ion trap.[25]

Bioinformatics. The LTQ-Orbitrap raw files were processed into Mascot Generic Files (mgf-files) using DTA Supercharger (http://msquant.sourceforge.net/). The resulting mgf-files were searched using an in-house MASCOT server (version 2.2) against the SwissProt *Homo sapiens* database (Sprot 52.3a, 264492 sequences; 96880444 residues). The glycosidase-treated samples were analyzed in 3 triplicate experiments and the mgf-files were searched using MASCOT Daemon version 2.2. The following search parameters were applied: *Enzyme*: Trypsin; *Fixed modifications*: Carbamidomethyl (C); *Variable modifications*: Acetyl (Protein N-term), deamidated (NQ), oxidation (M), phospho (ST), phospho (Y); *Mass values*: Monoisotopic; *Protein Mass*: Unrestricted; *Peptide Mass Tolerance*: ± 5 ppm; *Fragment Mass Tolerance*: ± 0.6 Da; *Max Missed Cleavages*: 2; *Instrument type*: ESI-TRAP.

Peptides with a MASCOT score probability of $p < 0.05$ was regarded as significant. All phosphopeptides were manually validated. During the manual validation, we investigated the intensity of the b and y fragment ions and the proline-directed fragmentation.

The *in silico* prediction of serine, threonine, and tyrosine phosphorylation sites in the identified phosphopeptides was performed using the neural network NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/).[26] The prediction gives a probabilistic p-score of the site being phosphorylated, where a *p*-value close to 1 indicates very high probability of phosphorylation.

RESULTS AND DISCUSSION

The protein concentration of CSF (500 μg/mL) is less than 1% of the total protein concentration in serum. When analyzed directly without prior concentration, very weak MS signals were obtained from CSF, and the titanium dioxide purification reproducibility was poor presumably due to the very large sample volume that had to be loaded onto these columns (data not shown). To increase the protein concentration and simplify the titanium dioxide purification step, the CSF samples were concentrated 70× using low-mass cutoff spin filters. As an alternative, direct drying of the CSF samples in a centrifugal vacuum evaporator was also tested and generally gave very similar results when analyzed by LC-MS/MS (results not shown). However, large amounts of salts and difficulties in solubilizing the dried proteins precluded the further use of this approach and all subsequent experiments were performed using CSF preconcentration on 3 kDa molecular weight cutoff spin filters. This preconcentration procedure using low mass cutoff spin filters, although enabling the identification of many novel phosphoproteins, causes the loss of peptides below the cutoff value.

The CSF tryptic digests were examined by MALDI-TOF MS (Figure 2a). The majority of peaks were in the 500−3000 m/z range and more than 100 peaks were easily detected in this m/z range.

The reduced, alkylated, trypsinated, and deglycosylated CSF samples were purified by TiO_2 chromatography and analyzed by MALDI-TOF MS (Figure 2). Although some phosphopeptides were found using this procedure, we found that the reproducibility of the tryptic digest of CSF, as well as the total number of phosphorylated peptides identified, increased when the CSF sample was predigested with Lys-C prior to trypsin before applying the TiO_2 purification strategy (data not shown).

The MALDI-TOF reflector-mode spectra were inspected for the metastable loss of H_3PO_4. Peptides containing phosphoserine and phosphothreonine residues may give rise to a broad low-resolution peak below the phosphopeptide peak[27,28] corresponding to the loss of phosphoric acid, especially if they are highly abundant

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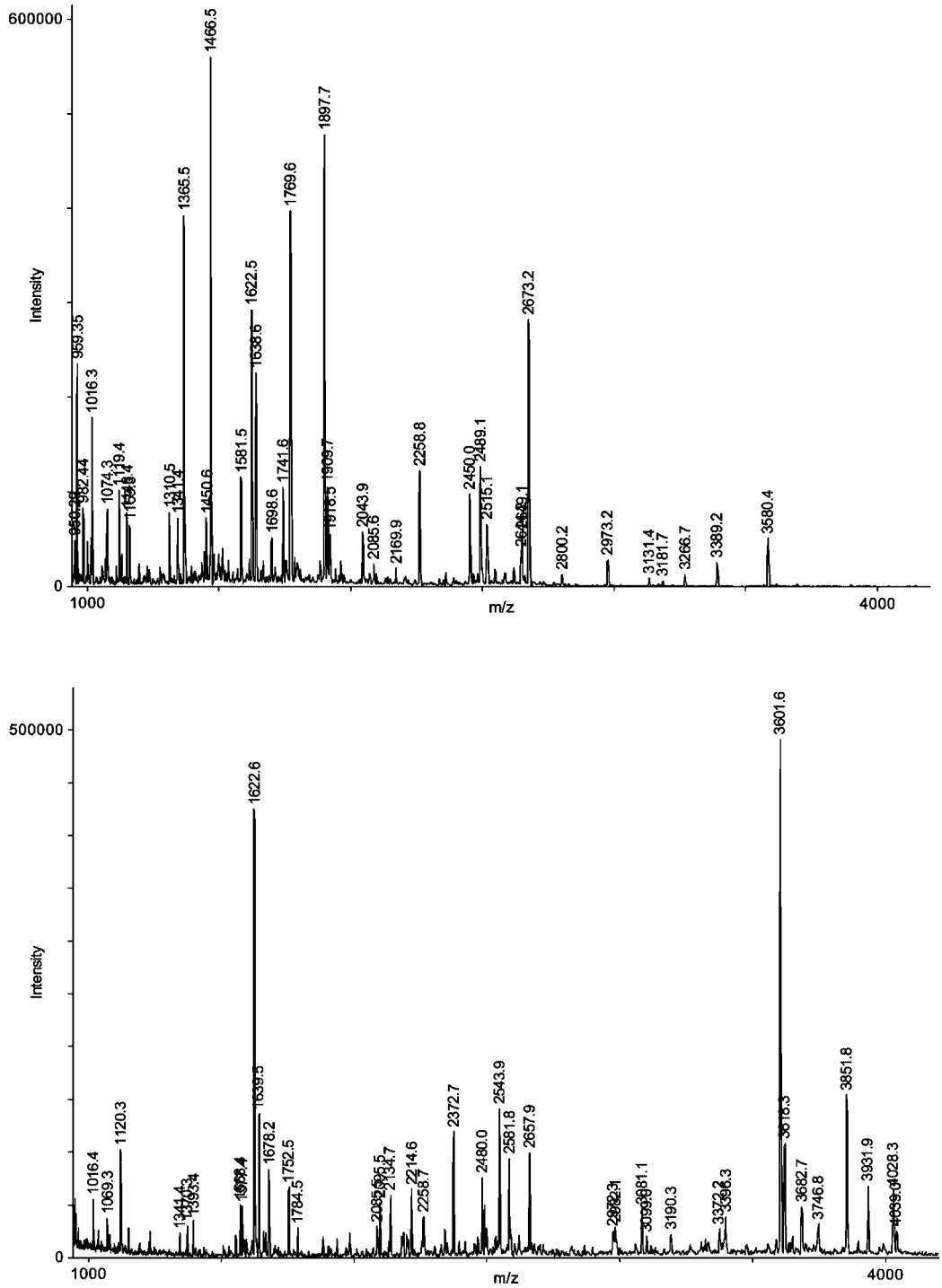

Figure 2. MALDI-TOF MS spectra of the concentrated and digested (top) CSF prior to and (bottom) after TiO_2 purification.

in the spectrum. However, no visible metastable ions were detected. This could be due to the absence of metastable fragmentation of the phosphopeptide, suppression of signal from the phosphopeptide owing to the negatively charged phosphate group on the peptide, the sheer absence of phosphopeptides in the sample, or simply the low abundance of these species in the sample.

To investigate whether the absence of metastable ions was due to the absence of phosphopeptides, the TiO_2 purified samples were analyzed on the more sensitive and sequencing-capable LC-MS/MS system. In this case, we identified phosphopeptides from 44 different phosphorylated CSF proteins (Table 1). Thirty-eight of the 44 proteins contained phosphorylation sites not previously described in the literature (Table 2). Fifteen putative phosphorylation sites on 11 different peptide fragments originating from the human neuroendocrine secretory granule protein secretogranin (Sg) 1 were identified. Although the phosphorylation of Sg 1 has previously been described,[29] our MS/MS data support additional phosphorylation sites at Ser-130, Ser-235, Ser-239, and Ser-245 residues in addition to the previously described phos-

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Table 1. Cerebrospinal Fluid Phosphoproteins Identified by LC-MS/MS after Titanium Dioxide Purification

protein	accession nr (Swiss-Prot)
Osteopontin	P10451
Secretogranin 1	P05060
Dickkopf related protein 3	Q9UBP4
SPARC-like protein 1	Q14515
Alpha 2 HS glycoprotein	P02765
Apolipoprotein E	P02649
Fibulin 1	P23142
Secretogranin 2	P13521
Kininogen 1	P01042
Chromogranin A	P10645
Fibronectin	P02751
Cystatin C	P01034
Inter alpha trypsin inhibitor heavy chain H2	P19823
Neuroendocrine protein 7B2 (Secretogranin 5)	P05408
Secretogranin 3	Q8WXD2
Trans Golgi network integral membrane protein 2	O43493
Receptor-type tyrosine-protein phosphatase N2	Q92932
Extracellular matrix protein 2	O94769
Nucleobindin 1	Q02818
Cadherin 2	P19022
Neuron navigator 2	Q8IVL1
Antithrombin III	P01008
Serum albumin	P02768
Versican core protein	P13611
Sushi domain-containing protein 5	O60279
Proenkephalin A	P01210
Cocaine- and amphetamine-regulated transcript protein	Q16568
Selenoprotein P	P49908
Apolipoprotein L	O14791
Fructose-bisphosphate aldolase A	P04075
Reticulocalbin-1	Q15293
Proprotein convertase subtilisin/kexin type 9	Q8NBP7
Matrix remodelling-associated protein 7	P84157
Amyloid precursor protein	P05067
Plasminogen	P00747
Follistatin-related protein 1	Q12841
Kallikrein-6	Q92876
Cofilin-1	P23528
Myrostoyl alanine-rich C-kinase substrate	P29966
Golgi phosphoprotein 2	Q8NBJ4
Latent-transforming growth factor beta binding protein	Q14766
Testican-3	Q9BQ16
Ubiquitin	P62988
Thy-1 membrane protein	P04216

phorylation sites. An example of the tandem MS fragmentation of the phosphorylated Sg 1 peptide 123−131 from CSF is shown in Figure 3. The Ser-130 was unambiguously assigned in Sg 1 123−131 phosphopeptide. The Sg 1 235−254 phosphopeptide was found to be both singly and doubly phosphorylated. When MS/MS spectra for the Sg 1 235−254 phosphopeptide were manually examined, the phosphorylation site could not be unambiguously assigned, although the phsophorylation of Ser-245 could be ruled out. This was due to the presence of several possible phosphorylation sites in the identified phosphopeptides and equivocal MS/MS spectra showing inconclusive b and y ion series of the peptide fragmentation. *In silico* prediction analysis showed almost equal probability of Ser-235, Ser-236, Ser-239, and Ser-245 being phosphorylated (Table 2).

Osteopontin, and alpha-2-HS-glycoprotein are well-known phosphoproteins and have been found in CSF in other studies.[4,30–32] In the present study, we identified several phosphorylated osteopontin peptides and found indications for three novel putative phosphorylated sites, i.e. on: Thr-227, Thr-237 and Ser-239. The phosphorylation sites could not be unambiguously assigned by manual inspection of spectra; however, the presence of a phosphate group on Thr-237 in the double phosphorylated peptide could be verified by manual inspection. Prediction analysis of phosphorylation revealed a much higher probability of Ser-239 being phosphorylated than Thr-227 and Thr-237 (Table 2).

Phosphorylation of kininogen and SPARC-like protein 1 in CSF has been reported in an earlier study where 2-D gel electrophoresis and peptide mapping was used, however, the exact phosphorylation sites on these proteins were not identified.[31] We have located the phosphorylations to Ser-84, Ser-92, Ser-198, Ser-272, Ser-295 and either Ser-413 or Ser-414 in SPARC-like protein 1. In kininogen, the precise phosphorylation site on the 325−343 peptide could not be delineated by a manual inspection of the MS/MS spectra however, fragment ions indicated that several sites could be phosphorylated. *In silico* prediction of phosphorylation showed that the likelihood of phosphorylation for Thr-327, Ser-329, and Ser-332 was equal (Table 2).

Completely novel phosphoproteins have also been identified in this study. To the best of our knowledge, the proteins Dickkopf-related protein 3, apolipoprotein E (ApoE), Apolipoprotein L, fibulin, Sg 2, Sg 3, antithrombin, trans-golgi network integral membrane protein 2, receptor-type tyrosine-protein phosphatase N2, extracellular matrix protein 2, cadherin 2, neuron navigator 2, serum albumin, versican core protein, sushi domain-containing protein 5, proenkephalin A, cocaine- and amphetamine-regulated transcript protein (CART), selenoprotein P, reticulocalbin-1, matrix-remodelling-associated protein 7, follistatin-related protein 1, kallikrein 6, golgi phosphoprotein 2, latent-transforming growth factor beta binding protein, testican-3, thy-1 membrane glycoprotein, and cystatin C (Figure 4) have not previously been reported to exist in phosphorylated isoforms. Interestingly, we have found a single phosphorylation site (Ser-82) on the human serum albumin that has never been described before even though this protein has been extensively studied in the past (Figure 5). Some of the phosphorylation sites in the above-mentioned proteins could not be annotated with absolute confidence after a manual analysis of the MS/MS spectra. However, *in silico* analysis of protein phosphorylation showed variable probability scores for the amino acids in question, making some of the putative phosphorylations more plausible than others (Table 2). Some of the putatively novel phosphoproteins give a very low probability score in the *in silico* prediction analysis for protein phosphorylation. As an example, the Ser-8 on neuron navigator 2 and Ser-258 on osteopontin can be mentioned (Table 2). This is most likely due to the fact that these prediction programs are trained on intracellular phosphorylation which might differ from that of extracellular phosphorylation as observed here.

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Table 2. Phosphorylated Amino Acid Residues in Phosphopeptides Purified by Titanium Dioxide and Identified by LC-MS/MS[a]

protein	peptide	sequence	residue	prediction (*p*-score)
Osteopontin	52−70	QNLLAPQNAVpSSEETNDFK		
	176−203	RPDIQYPDApTDEDITpSHMEpSEELNGAYK		
	204−220	AIPVAQDLNAPpSDWDpSR		
	223−241	DpSYETpSQLDDQpSAETHSHK	Thr-227	0.254
			Thr-237	0.125
			Ser-239	0.995
	250−268	ANDEpSNEHSDVIDpSQELpSK		
	269−290	VpSREFHpSHEFHpSHEDMLVVDPK		
	300−314	FRIpSHELDpSASpSEVN		
SPARC like protein 1	76−89	SKEESHEQSAEQGK	Ser-84	0.968
	90−98	SSSQELGLK	Ser-92	0.783
	187−220	DQGNQEQDPNISNGEEEEEKEPGEVGTHNDNQER	Ser-198	0.808
	256−286	MQEDEFDQGNQEQEDNSNAEMEEENASNVNK	Ser-272	0.934
	287−299	HIQETEWQSQEGK	Ser-295	0.559
	409−426	KAENSSNEEETSSEGNMR	Ser-413	0.981
			Ser-414	0.994
Secretogranin 1	123−131	WAEGGGHSR	Ser-130	0.995
	134−153	ADEPQWSLYPSDSQVpSEEVK		
	179−202	GEDpSpSEEKHLEEPGETQNAFLNER		
	235−254	SSQESGEEAGSQENHPQESK	Ser-235	0.987
			Ser-236	0.997
			Ser-239	0.998
			Ser-245	0.995
	259−276	pSQEEpSEEGEEDATSEVDK		
	305−324	GHPQEEpSEESNVSMASLGEK		
	372−387	APRPQpSEESWDEEDKR		
	397−409	MAHGYGEEpSEEER		
	617−640	pSAEFPDFYDpSEEPVSTHQEAENEK		
Secretogranin 2	257−269	IESQTQEEVRDSK	Ser-259	0.821
			Ser-268	0.998
	549−561	EHLNQGSSQETDK	Ser-555	0.381
			Ser-556	0.930
Receptor-type tyrosine-protein phosphatase N2	355−368	AALGESGEQADGPK	Ser-360	0.967
	428−448	SEHPESSLSSEEETAGVENVK	Ser-436	0.997
			Ser-437	0.997
			Thr-441	0.642
Dickkopf related protein 3	81−103	ASSEVNLANLPPSYHNETNTDTK	Ser-82	0.636
			Ser-83	0.848
Apolipoprotein E	138−152	GEVQAMLGQSTEELR	Ser-147	0.992
			Thr-148	0.116
Fibulin 1	147−163	SQETGDLDVGGLQETDK	Ser-147	0.917
Kininogen 1	325−343	ETTCSKESNEELTESCETK	Thr-327	0.943
			Ser-329	0.974
			Ser332	0.996
Cystatin C	35−51	LVGGPMDASVEEEGVRR	Ser-43	0.988
Secretogranin 3	35−53	ELSAERPLNEQIAEAEEDK	Ser-37	0.992
Trans Golgi network integral membrane protein 2	65−83	DSPSKSSAEAQTPEDTPNK	Ser-71	0.990
Extracellular matrix protein 2	209−227	EALQSEEDEEVKEEDTEQK	Ser-213	0.997
Cadherin 2	121−145	LSLKPTLTEESVKESAEVEEIVFPR	Ser-135	0.987
Neuron Navigator 2	1−9	MPAILVASK	Ser-8	0.059
Antithrombin III	61−78	KATEDEGSEQKIPEATNR	Ser-68	0.657
Serum Albumin	76−88	TCVADESAENCDK	Ser-82	0.758
Versican core protein	2108−2124	QEIESETTSEEQIQEEK.	Thr-2114	0.738
			Thr-2115	0.945
			Ser-2116	0.959
Sushi domain-containing protein 5	290−298	GSGEQQIMR	Ser-291	0.885
Proenkephalin A	237−252	FAEALPSDEEGESYSK	Ser-251	0.936
Cocaine- and amphetamine-regulated transcript protein	37−51	ALDIYSAVDDASHEK	Ser-48	0.982
Selenoprotein P	262−274	DMPASEDLQDLQK	Ser-266	0.839
Apolipoprotein L	306−320	VTEPISAESGEQVER	Ser-311	0.985
Fructose-bisphosphate aldolase A	29−42	GILAADESTGSIAK	Ser-39	0.031
Reticulocalbin-1	71−81	TFDQLTPDESK	Ser-80	0.974
Proprotein convertase subtilisin/kexin type 9	683−692	HLAQASQELQ	Ser-688	0.087
Matrix remodelling-associated protein 7	125−142	GPSSEGPEEEDGEGFSFK	Ser-127	0.995
			Ser-128	0.985
Amyloid precursor protein	439−450	VESLEQEAANER	Ser-441	0.907
Plasminogen	349−386	IPSCDSSPVSTEQLAPTAPPELTPVVQDCYHGDGQSYR	Ser-358	0.994

Table 2. Continued

protein	peptide	sequence	residue	prediction (*p*-score)
			Thr-359	0.243
Follistatin-related protein 1	163−170	LDSSEFLK	Ser-165	0.996
			Ser-166	0.075
Kallikrein-6	81−91	ESSQEQSSVVR	Ser-82	0.990
			Ser-83	0.997
Cofilin-1	2−13	ASGVAVSDGVIK	Ser-2	0.002
Myrostoyl alanine-rich C-kinase substrate	12−30	GEAAAERPGEAAVASSPSK	Ser-26	0.011
			Ser-27	0.867
Golgi phosphoprotein 2	253−267	EETNEIQVVNEEPQR	Thr-255	0.038
Latent-transforming growth factor beta binding protein isoform 1 L	1280−1297	GFVPAGESSSEAGGENYK	Ser-1287	0.791
			Ser-1288	0.881
			Ser-1289	0.752
Testican-3	33−42	SDGGNFLDDK	Ser-33	0.552
Ubiquitin	55−63	TLSDYNIQK	Thr-55	0.081
			Ser-57	0.880
Thy-1 membrane protein	88−97	VLYLSAFTSK	Thr-95	0.924
			Ser-96	0.794

[a] The *in silico* prediction of phosphorylation sites in the identified and not previously reported phosphopeptides are shown. Phosphorylation sites identified in this study are underlined. Previously reported phosphorylation sites have a "p" prefix and previously unknown phosphorylation sites are highlighted.

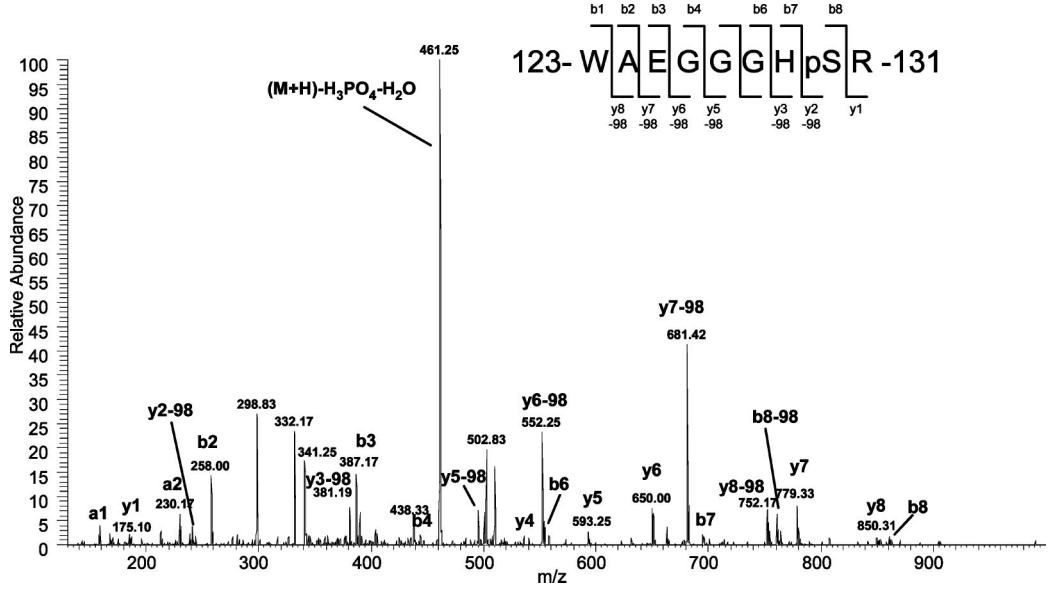

Figure 3. MS/MS spectrum of the phosphorylated secretogranin-1 peptide 123−131 from CSF. The MS/MS spectrum was obtained on an LTQ-Orbitrap after tryptic digestion and TiO_2 purification of phosphopeptides from the concentrated CSF. The spectrum shows the y and b ion series.

Protein phosphorylation regulates cellular signaling by modulating protein structure and function.[33] Dickkopf-related protein 3 has recently been shown to be strongly expressed in ganglioneuroma cells compared to neuroblastoma cells and could play a role as a potential marker for tumor maturation in neuroblastoma pathogenesis.[34] Moreover, markedly increased levels of kininogen fractions in CSF has been found in patients with neuropsychiatric lupus.[35] Apolipoprotein L1 has recently been suggested as a novel serum marker for liver fibrosis in patients with hepatitis C.[36]

CART functions as a satiety factor with anorexigenic properties.[37,38] The EM66 peptide, which encompasses the identified Sg 2 phosphorylated peptide, has been suggested to play a role in endocrine functions of the rat brain connected to food intake regulation.[39] Accordingly, Sg 3 single nucleotide polymorphisms

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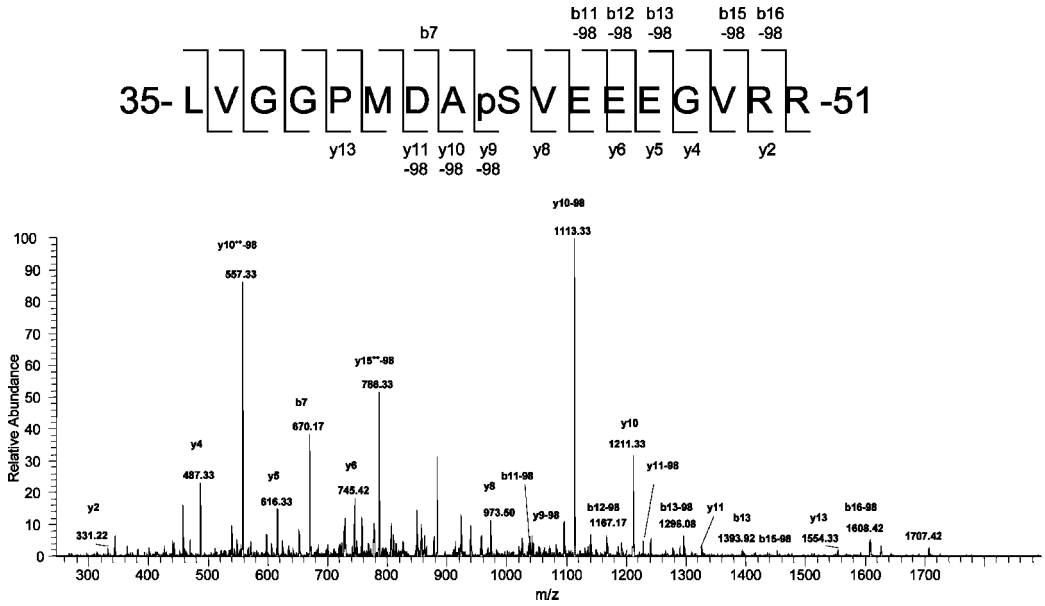

Figure 4. MS/MS spectrum of the phosphorylated cystatin C peptide 35−51 from CSF. The MS/MS spectrum was obtained on an LTQ-Orbitrap after tryptic digestion and TiO_2 purification of phosphopeptides from the concentrated CSF. The spectrum shows the y and b ion series.

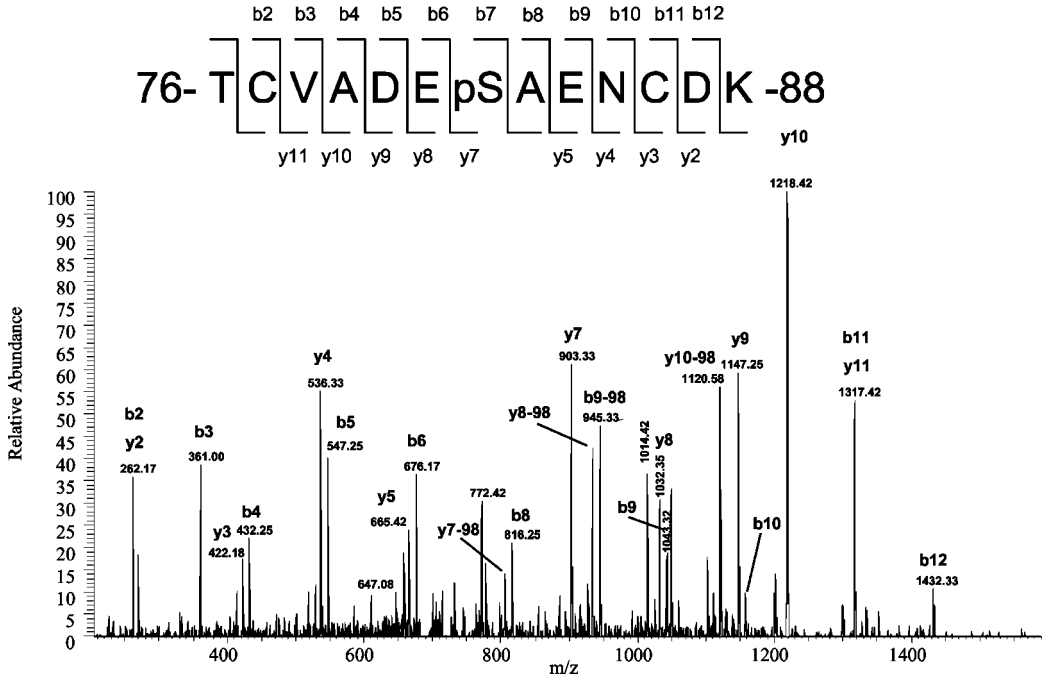

Figure 5. MS/MS spectrum of the phosphorylated serum albumin peptide 76−88 from CSF. The MS/MS spectrum was obtained on an LTQ-Orbitrap after tryptic digestion and TiO_2 purification of phosphopeptides from the concentrated CSF. The spectrum shows the y and b ion series.

have been shown to be connected with obesity[40] making Sg 2, Sg 3, and CART interesting candidates as markers for the regulation of food intake.

Amyloid precursor protein (APP) plays a central role in Alzheimer's disease (AD). Overexpression of APP as well as mutations in the *APP* gene result in the development of AD.[41–43] Cadherin 2 is an adhesion receptor in brain neurons found to interact with presenilin-1, which is a part of the gamma secretase complex that cleaves the amyloid precursor protein into the toxic amyloid beta 1−42 peptide found as amyloid aggregates in the

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brain of patients with AD. Kallikrein-6 has also been linked to the AD pathogenesis because decreased levels of kallikrein-6 are seen in the brain of AD patients.[44] Moreover, altered CSF levels of kallikrein-6 are seen in the AD group compared to controls.[45,46] Abnormalities of cystatin C production is also highly relevant in AD and have recently been found to modulate the risk of AD by affecting the amyloigenic properties of amyloid-β peptides.[47] Another protein of significant importance for AD is ApoE, which interacts with several of the pathological pathways in AD. Moreover, ApoE has been reported to be significantly altered in CSF from patients with AD.[48,49] In addition, it is hypothesized that the regulation of redox processes are not well regulated in AD, leading to oxidative stress in the brain of patients with AD.[50] Proteins acting as antioxidants, like selenoprotein 1,[51] may play a role in the redox processes in the brain. Phosphorylation of these AD-related proteins are interesting because of the apparent association of hyperphosphorylation with AD.[10] Further studies will show whether phosphorylation of these proteins are altered in AD patients compared to controls.

In addition to the novel identifications, the present study identifies many already known phosphorylation sites. Although the proteome of the CSF has been well studied during the last years,[4,5,8,49] the phosphoproteome in CSF is yet not well characterized. To the best of our knowledge, we have identified 56 novel phosphorylations in 38 CSF phosphoproteins, although we were not able to identify the well-characterized phosphoprotein tau in CSF, which is hyperphosphorylated in patients with AD.[52–54] This could be due to a low level of the phosphorylated tau isoforms protein in healthy individuals.

TiO_2 in general provide a selective enrichment that is around 90%,[55] however, this is sample dependent and lower selectivity can be observed for difficult biological samples such as body fluids. Here we obtained, in the best enrichments, more than 55% pure phosphopeptides (calculated as the number of phosphorylated peptides out of the total amount of peptides recovered in the LC-MS/MS run) which indicate that the enrichment procedure can be optimized further for such samples. Our results indicate that transferring the same purification method from experiments with blood and cell lysates to CSF is not straightforward. The inclusion of glycolic acid and highly acidic conditions in the peptide loading onto the TiO_2 columns should abolish binding of acidic peptides. However, we have found that the amount of TiO_2 used in the purification has to be matched with the amount of peptide material. If too much TiO_2 resin is used the selectivity decreases and we identify more nonphosphorylated peptides (Larsen, M.R., unpublished results).

Another obstacle in analyzing proteins in CSF is the presence of peptides from highly abundant proteins such as albumin in the peptide solution. The presence of these highly abundant peptides may not only interfere with the enrichment of phosphorylated peptides but also suppress the ionization of low-abundant phosphorylated peptides, making the identification of these species difficult. Therefore, depletion of the high abundant proteins would improve the detection of the low-abundant species. We have tried to deplete the most abundant proteins in CSF prior to the tryptic digestion and TiO_2 purification using two different depletion strategies: immunoaffinity column depletion of high abundant serum proteins and albumin depletion using an albumin specific stationary phase. In our hands, both methods depleted the CSF for most of its protein content and gave few protein identification hits (results not shown) indicating removal of essential proteins together with the treatments. This is partly in agreement with the study of Shores and Knapp,[56] who showed that depletion of high abundant proteins using immunodepletion or ultrafiltration (50 kDa cutoff) from CSF did not significantly improve the recovery of low-abundant proteins in CSF.

In conclusion, we have identified many novel phosphorylation sites on CSF proteins using TiO_2 chromatography and LC-MS/MS. Some of the phosphorylated proteins are known to participate in pathological processes in different diseases, for instance Alzheimer's disease; however, the relevance to these diseases awaits analysis of patient CSF. The TiO_2 phosphopeptide enrichment procedure to enrich for phosphorylated peptides was found to be less specific compared to previously reported studies[55] and studies performed in our group (Larsen, M.R., unpublished results); however, these experiments were done on total cell lysates and not on CSF. In addition to comparing the CSF-phosphoproteome enriched by this approach, future studies will also address methodological options to increase the amount of phosphopeptides detected by this method.

ACKNOWLEDGMENT

We thank Lene Jakobsen for excellent assistance in the laboratory. This work was partially funded by Lundbeckfonden and Gigtforeningen. M.R.L. was supported by a Young Investigator Award (Danish Strategic Research Council) and a SKOU fellowship (Danish Natural Science Research Council).

Received for review April 25, 2008. Accepted June 21, 2008.

AC800835Y

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