A novel isotopically labeled cysteine-tagging and complexity-reducing reagent, called HysTag, has been synthesized and used for quantitative proteomics of proteins from enriched plasma membrane preparations from mouse fore- and hindbrain. The reagent is a 10-mer derivatized peptide, H2N-(His)6-Ala-Arg-Ala-Cys(2-thiopyridyl disulfide)-CO2H, which consists of four functional elements: i) an affinity ligand (His6-tag), ii) a tryptic cleavage site (-Arg-Ala-), iii) Ala-9 residue that contains four (d4) or no (d0) deuterium atoms, and iv) a thiol-reactive group (2-thiopyridyl disulfide). For differential analysis cysteine residues in the compared samples are modified using either (d4) or (d0) reagent. The HysTag peptide is preserved in Lys-C digestion of proteins and allows charge-based selection of cysteine-containing peptides, whereas subsequent tryptic digestion reduces the labeling group to a di-peptide, which does not hinder effective fragmentation. Furthermore, we found that tagged peptides containing Ala-d4 co-elute with their d0-labeled counterparts. To demonstrate effectiveness of the reagent, a differential analysis of mouse forebrain versus hindbrain plasma membranes was performed. Enriched plasma membrane fractions were partially denatured, reduced, and reacted with the reagent. Digestion with endoproteinase Lys-C was carried out on nonsolubilized membranes. The membranes were sedimented by ultra centrifugation, and the tagged peptides were isolated by Ni2+/H11545 affinity or cation-exchange chromatography. Finally, the tagged peptides were cleaved with trypsin to release the histidine tag (residues 1–8 of the reagent) followed by liquid chromatography tandem mass spectroscopy for relative protein quantification and identification. A total of 355 unique proteins were identified, among which 281 could be quantified. Among a large majority of proteins with ratios close to one, a few proteins with significant quantitative changes were retrieved. The HysTag offers advantages compared with the isotope-coded affinity tag reagent, because the HysTag reagent is easy to synthesize, economical due to use of deuterium instead of 13C isotope label, and allows robust purification and flexibility through the affinity tag, which can be extended to different peptide functionalities.

The capability of comparing levels of individual proteins between two or more biological samples is rapidly becoming essential for all mass spectrometry (MS)1-based proteomics (1). A direct comparison of mass spectra intensities recorded in two separate measurements of two samples usually cannot easily be used for estimation of relative protein ratios. However, as the ionization efficiency of various isotopic forms of chemically identical peptides are the same, the spectral intensities can be used as a measure their relative abundances. Therefore, directed labeling of proteins or peptides with stable isotopes has been frequently used in quantitative proteomics. Considering the way in which the stable isotope label is introduced into an investigated sample, the labeling methods can be divided into two types: the metabolic and the sample post-processing ones.

Metabolic labeling of cultured cells is a classical approach already used half a century ago. For purposes of MS, either the whole media have been labeled with 15N (2–4) or the media were supplemented with amino acids containing 2H or 13C isotopes, first for improving specificity in database searching (5–8), and then for quantitative proteomics (9–11). The sample post-processing approaches are mainly based on derivatization of the thiol-moiety of cysteines (12) or acylation (13,14) with reagents carrying isotope labels. Alternatively, up

1 The abbreviations used are: MS, mass spectrometry; LC, liquid chromatography; MS/MS, tandem mass spectrometry; ICAT, isotope-coded affinity tag; SCX, strong cation exchange; DPDS, 2,2'-dipyridyl disulfide; IMAC, immobilized metal affinity chromatography; Fmoc, 9-fluorenylmethoxycarbonyl; DTT, dithiothreitol; GB, gradient buffer; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; CID, collision-induced dissociation.
to two $^{18}$O atoms can be incorporated into carboxyl groups of peptides by digestion with trypsin or another endopeptidase in the presence of $\text{H}_2\text{O}^{18}$O (15–17).

In order to identify and quantify proteins in complex samples, isotope labeling in combination with affinity selection (18, 19) has proven to be an especially successful tool. Iso- tope-coded affinity tags (ICATs) with a normal (H)- or stable heavy isotope-enriched (D)-labeled biotin reagent have been successfully applied for quantitative profiling of differentiation-induced microsomal proteins from human myeloid leukemia cells (20) and for quantitative analysis of Myc oncoprotein function (21).

In this study, we describe a novel tool for identification and quantification of cysteine-containing peptides in complex peptide mixtures: the HysTag reagent. This reagent is a decapetide of the sequence (H)$_6$ARAC that is activated with 2,2-dipyridyl disulfide (DPDS), which selectively makes the HysTag reagent reactive toward cysteine side chains. In the “heavy” form of the reagent, Ala-9 contains four deuterium atoms. The amino-terminal histidines are the tag that enables selective isolation of tagged peptides by metal-affinity or ion-exchange chromatography. Subsequent digestion of tagged peptides with trypsin releases the H$_6$AR portion of the HysTag from the peptide that is covalently bound via the disulfide bridge with the isotope-labeled dipeptide AC (see Fig. 1).

To demonstrate its applicability for analysis of complex mixtures we used the HysTag for identification and relative quantification of proteins from two distinct areas of mouse brain. Fractions enriched in plasma membranes were prepared from fore- and hindbrain, and the proteins were labeled with the reagent direct on the membranes either with heavy or light reagents. The membranes from both brain compartments were mixed and treated with HysTag and digested with Lys-C. Following cation exchange chromatography and digestion with trypsin the released isotope-coded peptides were analyzed by liquid chromatography (LC)-microcapillary electrospray ionization tandem mass spectroscopy (MS/MS). A total of 355 unique proteins were identified, among which 281 could be quantified.

**EXPERIMENTAL PROCEDURES**

**Solid-phase Synthesis of Isotope-labeled HysTag Peptides**—The 10-mer HysTag peptides, light and heavy forms, were made by automated solid-phase peptide synthesis on a prototype Peptide Synthesizer (Intavis Bioanalytical Instruments, Cologne, Germany). Pre-loaded (Fmoc)-Cys(Ttr)-NovASyn® TFA-label and Wang-type amino resin was coupled with 9-fluorenylmethoxycarbonyl (Fmoc)-protected alanine (Novabiochem, Lauffelfingen, Switzerland) using benzotriazol-1-yloxy-tris-pyrrolidino-phosphonium hexafluorophosphate (BOP)/H$_2$N, and the cleaved peptide was precipitated in 10 ml ice-cooled tert-butylmethyl ether as a white milky solution. The solution was centrifuged for 10 min (1000 rpm) and the ether-phase decanted to waste. The precipitated peptide was redissolved in water with 5% formic acid. Nano-electrospray MS and MS/MS spectra of the peptide were acquired as control of purity.

**Activation of the HysTag Peptide**—The crude peptide solution was lyophilized and redissolved in 0.1 M Tris-HCl, pH 8.0. Dithiothreiotel (DTT) was added to a final concentration of 10 mM, and the peptide solution was incubated for 30 min at room temperature to reduce potential disulfide bridges. The peptide solution was then reacted with 10 mM excess of DPDS in 50% (v/v) acetonitrile for 1 h. Finally, the derivatized peptides were purified by reverse-phase chromatography on a C$_{18}$ column and lyophilized.

**Preparation of Crude Cell Membranes**—Adult mice were sacrificed by decapitation and brain material was dissected in less than 30 s. Fore- and hindbrain were separated and rinsed with PBS. Approximately 300 mg brain tissue (one forebrain or three hindbrains) was manually ground in 5 ml gradient buffer (GB) buffer containing 0.32 M sucrose, 10 mM HEPES-NaOH, 100 mM succinic acid, 1 mM EDTA, pH 7.4, 0.25 mM DTT, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 20 M leupeptin hemisulfate, 150 M aprotinin in a 15-ml glass potter on ice and the “initial homogenate” was centrifuged at 1000 $\times$ g for 10 min. The supernatant was discarded, and the pellet was homogenized in 4 ml GB buffer using an IKA Ultra Turbax blender (IKA-Ultra Turrax, Staufen, Germany) at maximum speed for 5–10 s (the “homogenate”). The suspension was centrifuged as above, and the supernatant was collected. The rehomogenization process was repeated twice, the supernatant pooled, and crude membranes collected at 50,000 $\times$ g for 30 min. The pellet (P2) was resuspended in 4 ml GB buffer with five strokes of the motorized Potter homogenizer (B. Braun Biotech, Melsungen, Germany). The suspension was sonicated twice on ice for 15 s using Soniprep 150 (Sanyo, Gallenkamp, UK).

**Preparation of Enriched Plasma Membranes by Density Gradient Centrifugation**—In a 11.5-ml crimp tube (S/L, tube PA, 11.5 ml; Sorvall, Asheville, NC), the resuspended P2 fraction was mixed with 3.85 ml 100% Percoll (Amersham Biosciences, Piscataway, NJ) and 0.6875 ml 2 M sucrose. The tube was filled with GB buffer, closed, and centrifuged at 50,000 rpm in a fixed-angle rotor T 890 centrifuge (Sorvall) at 4°C for 15 min. The gradient was fractionated from the top by the displacement method. Percoll was removed by centrifugation of the fractions in 1-ml PC tubes in Sorvall RC M150 GX using the S150AT rotor at 900,000 $\times$ g for 4°C for 20 min.

The composition of individual fractions was analyzed for $\gamma$-glutamyl transpeptidase (marker for plasma membranes) (22), cytochrome c oxidase (marker for mitochondria) (23), and NADPH-cytochrome c reductase (marker for endoplasmatic reticulum) (24) activities and total protein content using DC protein kit (Bio-Rad, Hercules, CA). The yield of plasma membranes was 2–3 mg protein/g brain.

**Labeling of Standard Proteins**—Fifty micrograms of bovine serum albumin (BSA) and human Transferrin were reduced with 50 mM DTT at room temperature for 0.5 h. Following desalting of an HiTrap desalting 5-ml column (Amersham Biosciences), the proteins were incubated in the presence of 100-fold molar excess of either Hys-Tag-d$_2$ or Hys-Tag-d$_3$ in 150 $\mu$M of 4 M urea in 0.1 M Tris-HCl, pH 7.8, at room temperature for 4 h. The “light” and “heavy” samples were mixed and incubated with 1 $\mu$g of endoproteinase Lys-C at room temperature overnight.

**Labeling of Membrane Proteins**—The method comprises repeated
steps of membrane incubation and separation of the “solid phase” membranes from “liquid phase” by ultra centrifugation. During all incubations, the samples were gently mixed. The “phase” separations were achieved by centrifugation at 150,000 × g rpm in 4 °C for 15 min using RC M150 GX centrifuge and the S150AT rotor. Fore- and hindbrain membrane fractions containing ~1 mg total protein were pooled, and the membranes were collected by centrifugation at 150,000 × g rpm in 4 °C for 15 min using RC M150 GX centrifuge and the S150AT. The pellets were reuspended in 400 μl of 0.2 M NaBr, 0.2 M KCl, 10 mM DTT, 50 mM Tris-HCl, pH 8.0. After 30 min incubation at room temperature the membranes were sedimented by centrifugation. Then the pellets were reuspended in 200 μl of 4 mM urea in 0.1 M Tris-HCl, pH 8.0, and divided into two equal fractions. The fractions were mixed with 0.8 mg of either HysTag-d6, or HysTag-d2, and incubated at room temperature for 4 h. The membranes were collected by centrifugation (as above). “Heavy” and “light” membranes were mixed in a ratio 1:1 and digested with 5 μg of endoproteinase Lys-C at room temperature overnight. Following the next centrifugation, the supernatants were collected and the pellets discarded.

Isolation of Tagged Peptides Using Cation Exchanger—Two hundred microilters Source 30S gel slurry (Amersham Biosciences) was pipetted into a 1-ml spin columns (type size) and centrifuged at 700 × g for 1 min. The columns were washed with 2 × 500 μl elution buffer (25 mM 4-morpholinoepanesulfonic acid-NaOH, 1 mM NaCl, pH 7.0) and equilibrated with 3 × 500 μl binding buffer (8 mM urea, 25 mM 4-morpholinoepanesulfonic acid (MES), pH 5.5). The Lys-C digests were diluted 5-fold with the binding buffer to a final volume of 1.0 ml, and the pH was adjusted to 5.5 with 1 M HCl. The diluted samples were loaded into the spin columns, and incubated at room temperature for 1 h. After incubation, the spin columns were placed in 2 ml Eppendorf (Hamburg, Germany) tubes and centrifuged at 700 × g for 1 min, and the resin was washed twice with 500 μl 25 mM MES, 0.1 M NaCl, 1% 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid (CHAPS), pH 5.5, followed by 2 × wash with 25 mM MES, 0.1 M NaCl, pH 5.5. Finally, the bound peptides were eluted with 2 × 100-μl elution buffer.

Isolation of Tagged Peptides Using Immobilized Ni2+—Specific His6-Tag IMAC purification was performed using the nickel-chelated B-PER His6 spin column kit according to the manufacturer’s instructions (Pierce, Rockford, IL). Briefly, 1 ml of precharged nickel-chelated spin columns were washed and equilibrated with 2 × 2 ml of B-PER Reagent. The Lys-C digests were diluted 10-fold with the B-PER Reagent to a final volume of 2 ml and applied to the spin column. After 1 h incubation at room temperature, the spin columns were placed in 2 ml Eppendorf tubes and centrifuged at 700 × g for 1 min and the resin was washed three times with 1 ml of B-PER washing buffer supplemented with 1% CHAPS to remove nonspecific-binding hydrophobic peptides. Finally, the bound peptides were eluted with 2 × 500 μl B-PER elution buffer (2% imidazole w/w solution).

Tryptic Digestion of the Tagged Peptides—One microgram trypsin (Modified Sequence Grade; Promega, Madison, WI) was added to each peptide eluate and incubated overnight at 37 °C. The tryptic peptide mixtures were desalted and concentrated on Poros R2/oligo R3 (1:1) resins (Perceptive Biosystems, Foster City, CA) packed in Geloader tips (Eppendorf) as described (25) and eluted in 2 × 2 μl (50% MeOH, 5% HCO2H) into 96-well plates, where they were diluted to a final volume of 20 μl (10% MeOH, 5% HCO2H).

MS: Microcapillary LC-MS/MS Analysis—Microcapillary reverse-phase high-performance LC-MS/MS was performed using an Agilent 1100 capillary LC system with an μ-Autosampler (Agilent Technologies Inc., Palo Alto, CA) coupled to a QSTAR Pulsar hybrid quadrupole-time-of-flight mass spectrometer (AB-MSD Sciex, Toronto, Canada) using a modified nano-electrospray ion source (Proxene Biosystems, Odense, Denmark) interface.

The tryptic peptide mixtures were auto-sampled at a flow rate of 5.0 μl/min onto a precolumn (150 μm id × 3 cm fused silica; Composite Metal Services, West Yorkshire, UK) in-house packed with C18 material (Zorbax C18 5-μm particles; Agilent Technologies) and then eluted with a linear gradient of H2O-MeCN in the presence of 0.4% acetic acid plus 0.005% heptfluorobutyric acid at a flow rate of 0.3 μl/min to 40% MeCN for 90 min. The precolumn eluate was separated on an analytical capillary C18 column (Zorbax C18 3.5-μm particles; Agilent Technologies) packed in a pulsed fused silica capillary emitter (75 μm id × 8 cm; New Objectives, Cambridge, MA) mounted in the nano-electrospray ion source. A voltage of 2.0 kV was applied behind the emitter through a platine wire into one arm of the microcross T (Upchurch Scientific, Oak Harbor, WA) connecting the precolumn with the analytical column packed in the emitter.

The mass spectrometer was operated in the information-dependent acquisition mode to automatically switch between MS and MS/MS acquisition controlled by the Analyst software. Survey MS spectra were acquired for 1 s with doubly, triply, and quadruply charged ions triggering the function switching (MS→MS/MS). The most intense ion was isolated and fragmented for 2 s by low-energy collision-induced dissociation (CID) MS/MS. The collision energy was automatically calculated and adjusted for each CID-MS/MS spectra individually. Former target ions were dynamic excluded for 180 s. Both MS and MS/MS spectra were acquired with the Q2-pulsing function switched on and optimized for optimal transmission of ions in the sequence tag mass region (m/z 400–1000).

Database Searches: Peptide Identification—All MS/MS spectra files from each LC run were centroided and merged to a single file, which were searched using the Mascot Search Engine (Matrix Science, London, UK) against the mammalian NCBI database with oxidized methionine (+15.99 Da) and HysTag-d6 and -d2 cysteine (+186.04 Da and +194.07 Da, respectively) as variable modifications. Searches were done with initial tolerance on mass measurement of 1.3 Da in MS mode and 0.13 Da in MS/MS mode. The rather large mass tolerance in MS mode was used to ensure identification of peptide ions selected and isolated by their 13C isotope instead of the 12C isotope via the Analyst software.

RESULTS AND DISCUSSION

The Reagent and Its Properties

The HysTag Labeling and Quantification Procedure—One of the major challenges of proteomics is quantification of the individual proteins in complex mixtures. This is a difficult task because of the complexity and dynamic range of proteomes. To address this problem, we have designed and tested a new protein identification and quantification tool, HysTag. The reagent is a derivatized decapeptide, H2N-(His)6-Ala-Arg-Ala-Cys(2-pyridyl disulfide)-CO2H, which consists of four functional elements: i) an affinity ligand (Hys6-tag), ii) a tryptic cleavage site (-Arg-Ala-), iii) an Ala-9 residue that contains four (d9) or zero (d0) deuterium atoms, and iv) a thiol-reactive group (DPDS).

The HysTag procedure consists of three main steps (Fig. 1): 1) covalent tagging of reduced and desalted protein samples with HysTag, followed by endoproteinase Lys-C digestion of the combined labeled protein samples; 2) isolation of tagged peptides by charge-dependent strong cation exchange (SCX), or 3) immobilized Ni2+ ion affinity chromatography (IMAC) followed by tryptic digest of the isolated peptides. Identification and quantification of the tagged tryptic peptides was
determined by microcapillary LC-MS/MS. SCX isolation of the tagged peptides is based on the strong cationic properties of the HysTag. The imidazole ring of histidine side-chain has a \( pK_a \) value of 6.2 (26), therefore at a pH below 6.2 the His-tag will have a net positive charge. This provides a sufficiently high density of positive charges to allow strong binding to a cation exchanger at 5.0 < pH < 6.0, whereas the majority of untagged peptides generated by Lys-C proteolysis have a net negative charge at pH > 5 (pl < 5). This therefore allows selective purification of His\(_6\)-tagged peptides from undesired nontagged peptides.

**Chromatographic and Mass Spectrometric Properties of Tagged Peptides**—To analyze the chromatographic separation of HysTag-modified peptide pairs, human Transferrin and BSA were labeled with heavy and light HysTag reagent and processed as described above. The elution profiles from four pairs of the HysTag-labeled peptides originating from BSA and Transferrin pairs are shown in Fig. 2. Co-elution of the d\(_0\)- and d\(_4\)-labeled forms was observed for each peptide pair in all LC-MS runs. This property of the reagent simplifies the quantification procedure and increases the accuracy of quantification. The chromatographic behavior of the tagged peptides is unique, because usually deuterated peptides and the corresponding nondeuterated peptides do not co-elute (27). The expected chromatographic isotope effect is probably diminished due to the adjacent hydrophilic groups (28).

It is a key feature of our method that a relatively long peptide with desired retention behavior is used for peptide isolation, whereas the relative small remaining mass added to the cysteine residue after trypsin digestion makes the CID-MS/MS spectra “clean” and easy to interpret. Removal of the His\(_6\)-tag after affinity purification but before LC-MS generates...
MS/MS spectra that predominantly display b- and y-type fragment ions, whereas no abundant interfering losses from cleavage of the HysTag label are observed (Fig. 3). This makes the MS/MS spectra easy to identify via standard database search algorithms with cysteine specified as modified by the dipeptide.

Specificity and Efficiency of the Method—The efficiency of derivatization and the specificity of peptide selection was tested on purified human transferrin and BSA. Each of these standard proteins was reduced and derivatized with “heavy” and “light” HysTag reagent and mixed in a ratio of 1:1. Following the affinity separation and digestion, the samples were analyzed by microcapillary LC-MS/MS. The analysis of transferrin and BSA enabled identification of 15 from 27 cysteine-containing peptides in transferrin and 19 from 25 cysteine-containing peptides in BSA peptide pairs (d0:d4) (not shown). The d0:d4 ratios of each BSA and transferrin peptide pair were 1.060 ± 0.049 and 0.988 ± 0.052, respectively. This is comparable to results reported recently (29) for an improved, cleavable ICAT reagent.

Reduction of Sample Complexity—We next verified the utility of the HysTag procedure for the identification of a maximum number of proteins in very complex samples. A MCF-7 breast cancer cell cytosol fraction was analyzed by single microcapillary LC-MS/MS runs (data not shown), once after simple reduction and alkylation by iodoacetamide and once after reduction, coupling to HysTag reagent and selection of Cys-containing peptides. Mascot search of the microcapillary LC-MS/MS run of the HysTag MCF-7 cytosolic sample identified 152 unique proteins. In contrast, the normally processed sample enabled identification of only 54 unique proteins.

Differential Display Analysis of Enriched Plasma Membrane Preparations from Mouse Fore- and Hindbrain

Despite the growing importance and interest in brain biology, and its age-related degenerative processes, little proteomic effort has been devoted to the analysis of the plasma membranes of the brain. To demonstrate the practical applicability and potential of the HysTag strategy on this difficult to analyze tissue, we performed an initial survey of the quantitative differences between plasma membranes from mouse fore- and hindbrains.

Isolation of Plasma Membranes—Plasma membranes represent only 2–5% of the total membrane component of eukaryotic cells. The most abundant membranes are those from

![Fig. 2. MS survey spectra and LC elution profiles (extracted ion current, XIC) of a BSA peptide (left) and a transferrin peptide (right) labeled with heavy and light HysTag reagent. The XIC for all peptide pairs demonstrates co-elution; this is an important feature of this HysTag reagent, because quantification of the pairs is readily obtained from their relative ratios in the MS survey spectra without the need for 13C reagents.](image-url)
the rough endoplasmatic reticula and mitochondria, representing 35–60% and 21–39% of all membranes, respectively. As a consequence, efficient isolation of a fraction enriched in plasma membrane is a prerequisite for identification of cell-surface proteins.

Isolation of the plasma membrane from brain is complicated by the presence of large quantities of mitochondria and myelin (30). Therefore, standard mass spectrometric analysis of crude membrane pellets resulted in identification only a small number of plasma membrane and/or integral membrane proteins (data not shown). As a high content of mitochondrial proteins and other nonmembranous proteins were characteristic of these samples, it was necessary to fractionate the crude membrane pellet by density gradient centrifugation to specifically enrich for plasma membrane proteins. Forty percent Percoll in 0.32 M sucrose was used as a gradient medium, and samples were separated into 12 fractions. For quantitative monitoring of the distribution of plasma membrane, mitochondria, and endoplasmatic reticulum, organelle-specific marker activities were determined for each fraction. Addition of succinate at the beginning of the fractionation procedure was essential to increase the buoyant density of mitochondria. This method has previously been reported for pancreatic mitochondria (31). Up to a 10-fold enrichment of plasma membrane was observed in the top fractions of the gradient (fractions 1–5; Fig. 4). Fractions 1–5 were substantially depleted of mitochondria and endoplasmatic reticula (Fig. 4). The yield of the plasma membrane marker \(y\)-glutamyl transpeptidase was \(\sim 10\%\) in each of the gradient-top fractions, and therefore four to five of the fractions used for isolation of membrane proteins contained \(\sim 40\%\) of the plasma membrane present in the crude membrane pellet (Fig. 4).

**Processing of Plasma Membranes—**Solubilization of membrane proteins is a key issue in membrane biochemistry (32). Typically, proteins are extracted from the phospholipid bilayer into detergent micelles that maintain membrane proteins in solution in their biologically active form and enable fractionation of these proteins using various separation techniques. In proteomic approaches dedicated to the identification of proteins, preservation of biological activity is not important and therefore usually SDS, a strongly denaturing detergent, is...
used (20, 33). Because even at lower concentrations detergents affect mass spectrometric analysis, they have to be removed from the analyzed samples. While polyacrylamide electrophoresis is used as the fractionation method, the detergent is removed by extensive washing of the gels with alcohol-containing solutions. SDS can also be successfully removed from samples by cation exchange chromatography in the presence of moderate concentration of organic solvent (20), but we have found that even successful depletion of the detergent and efficient digestion still leads to difficult-to-analyze samples. As a consequence of these considerations, we have developed a method that at an early step of sample preparation separates hydrophilic peptides derived from protein domains outside the membranes from those that are embedded in the membranes (Fig. 5). The proteins are denatured, reduced, and modified with HysTag reagent directly on the membranes. This strategy is facilitated by the fact that many membrane proteins contain disulfide bridges to stabilize their extracellular domains. Following digestion with endoproteinase Lys-C, the membranous “solid phase” was separated from the released peptides by ultracentrifugation. The isolated fraction was directly subjected to affinity selection of tagged peptides. To achieve precise and unambiguous identification and quantification of the analyzed proteins, two inverse labeling experiments (16) are performed in parallel for each sample. Either the forebrain membranes were labeled with $d_4$ reagent and mixed with $d_0$-labeled hindbrain or the membranes for the forebrain were modified with $d_0$ reagent and were mixed with $d_4$-labeled membranes for hindbrain. After completion of this study, a different strategy, which also involves “on membrane” digestion, has been applied to the nonquantitative mapping of membrane proteins (34).

**Fig. 4.** Purification of plasma membranes from fore- (A and B) and hindbrain (C and D). Pellets containing crude membranes were separated in Percoll density gradients. The gradients were fractionated, and the fractions were assayed for enzymatic marker activities and protein content. The data are mean values from four independent experiments. Solid lines, plasma membrane marker activity; short-dashed lines, mitochondrial marker activity; dotted lines, endoplasmatic reticulum marker, long-dashed lines, total protein. The enrichment and yield values refer to the crude membrane pellet. Fractions 1 and 12 are from the top and bottom of the gradient, respectively.

**Differential Display Data and its Statistical Evaluation**—For an initial overview of the quantitative differences between membrane proteins in mouse fore- and hindbrain, the above procedure was performed on two different mouse brain samples. To check the complete procedure, four forward and four inverse analyses were performed. These resulted in identification of 1263 unique peptides representing 355 unique proteins (Table I). Of these, 281 proteins could be quantified.
Approximately one-fifth of them were proteins annotated as plasma membrane components (not shown), supporting the efficiency of the purification protocol even for this type of low-abundant membranes. The membrane plasma proteins quantified in this study belong to distinct families including ion channels, solute carriers, vesicle transport system, adhesion molecules, and others. Channels gated by voltage and ligands were identified, as were channels functioning as excitatory (glutamate) and inhibitory (γ-aminobutyric acid, GABA) receptors (Table II). For most of the identified proteins, moderate differences in levels between fore- and hindbrain were observed (0.5–2), but some proteins differed by larger ratios. Below, we discuss a few of these.

Glutamate is the main excitatory neurotransmitter in the brain. To date, five glutamate transporters have been cloned: GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5. These transporters are believed to be critical in reducing potentially toxic extracellular concentration of glutamate by rapid uptake into nerve terminals and glial cells. In this study, we identified two of the glutamate transporters, GLAST and GLT1. Quantification revealed that GLAST is 2.7 times more abundant in hindbrain, whereas GLT1 levels are similar in both brain regions (hind-/forebrain ratio, 0.83; Table II). This result correlates well with previously identified high levels of GLAST in adult rat cerebellum (35, 36), the main part of the hindbrain.

Another important finding is the distribution of the synaptotagmin variants I and II, a synaptic vesicle membrane protein thought to be a Ca2+ sensor for neurotransmitter release in mammalian brain. The levels of variant I are 2.4 times higher in the forebrain and levels of variant II are 3.8 times higher in the hindbrain (Fig. 6 and Table II). Again, these findings from quantitative proteomics correlate well with the fact that synaptotagmin I is preferentially expressed in cerebral cortex and hippocampus (forebrain) and synaptotagmin II is primarily expressed in cerebellum, spinal cord, and brain stem (37).

Elevated levels of myelin-oligodendrocyte glycoprotein and proteolipid protein (myelin) in hindbrain (Table II) can be explained by the higher extent of myelination of the regions of the caudal brain regions in comparison to the rostral ones.
reflecting well-known phylogenetic and developmental patterns of the mammalian brain.

The character of the quantitative brain data presented here is preliminary and serves only to demonstrate the potential of the HysTag strategy for realistic samples. However, more extended studies using the HysTag strategy involving addi-

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**TABLE II**

Selected membrane proteins quantified in the plasma membrane fractions from fore- (FB) and hindbrain (HB) in forward and reverse labeling experiments

| Swiss-Prot/TrEMBL | Protein name | FB/HB ratio | Δ SD | Identified peptide | FB-d0/ HB-d4 ratio | Δ SD | FB-d0/ HB-d0 ratio | Δ SD |
|------------------|-------------|-------------|------|-------------------|-------------------|------|-------------------|------|
| Ion channels and transport proteins |
| Q64436 | H+/K+ transporting ATPase, α chain | 1.60 | 0.19 | NLEAVETLGSVSCDVK | 1.51 | 0.19 | 1.69 | 0.08 |
| P14094 | Na+/K+ -transporting ATPase β-1 chain | 1.18 | 0.10 | YNPNVLPQCTGK | 1.31 | 0.03 | 1.12 | 0.06 |
| P14231 | Na+/K+ -transporting ATPase β-2 chain | 2.87 | 0.63 | DDMIFEDGCVNPSEP | 1.26 | 0.05 | 1.10 | 0.10 |
| P97370 | Na+/K+ -transporting ATPase β-3 chain | 0.88 | 0.09 | SCGQVVEEKW | 3.01 | 0.03 | 2.48 | 0.19 |
| P56564 | Excitable amino acid transporter 1 GLAST | 0.37 | 0.18 | NMFPPLNEACFK | 0.40 | 0.18 | 0.33 | 0.03 |
| P43006-1 | Excitable amino acid transporter 2 GLT1 | 0.11 | 0.16 | NLFVPNMLQACFQIQTVTK | 1.12 | 0.08 | 1.22 | 0.14 |
| P43006-3 | Excitable amino acid transporter 2 splice isoform Glt-1B | 1.31 | 0.08 | SADCVEEEEPWK | 1.37 | 0.11 | 1.01 | 0.03 |
| Q9R1C4 | Sodium bicarbonate cotransporter NBC1 | 0.49 | 0.25 | HVCDEEEVEGHHTIYYVHVK | 0.47 | 0.25 | 0.51 | 0.21 |
| Q912Q2 | Glycine transporter type 2 | 1.39 | 0.35 | LLLDSCVIGDHPR | 1.35 | 0.13 | ND | ND |
| O35633-1 | Vesicular inhibitory amino acid transporter | 0.74 | 0.01 | SEGECGEAEGAPVWGYQHR | 0.74 | 0.01 | ND | ND |
| P31650 | Sodium- and chloride-dependent GABA transporter 4 | 0.64 | 0.12 | TVTNDCAEAK | 0.58 | 0.10 | 0.69 | 0.12 |
| O35874 | Neutral amino acid transporter A Receptors |
| Q03137-1 | Ephrin-A4 receptor | 1.33 | 0.12 | NILVNSNLVCK | 1.25 | 0.12 | 1.28 | 0.12 |
| Q8K087 | G protein-coupled receptor 1 GLAST | 0.83 | 0.17 | RSLWASCQGVTVSEQRL | 0.85 | 0.17 | 0.82 | 0.05 |
| O55022 | Progesterone receptor membrane component 1 | 0.31 | 0.04 | GLATFCLDK | ND | ND | 0.31 | 0.04 |
| Synaptic vesicle proteins |
| Q64332 | Synapsin lib | 1.40 | 0.13 | DLYFEDMCSPMLGHEKHVD | 1.30 | 0.08 | 1.50 | 0.13 |
| P46096 | Synaptotagmin I | 2.40 | 0.11 | LGDCIFSLR | 2.50 | 0.05 | 2.30 | 0.11 |
| P46097 | Synaptotagmin II | 2.03 | 0.05 | LGDCITLSR | 0.28 | 0.12 | 0.24 | 0.09 |
| Q88G39 | Synaptic vesicle glycoprotein 2 | 1.66 | 0.13 | CYFEDVTSTDTYFK | 1.67 | 0.08 | 1.65 | 0.13 |
| Other proteins |
| P01831 | Thy-1 membrane glycoprotein | 1.44 | 0.16 | VTSLTAUCVLWNQLR | 1.51 | 0.16 | 1.36 | 0.04 |
| Q86L3K | Limbic system-associated membrane protein |
| Q8R464 | Membrane glycoprotein TSLC1-like 2 | 0.56 | 0.16 | VDVYVDEGTSYCTSVQVQHEPK | 0.74 | 0.08 | 0.73 | 0.03 |
| Q61885 | Myelin-oligodendrocyte glycoprotein | 0.31 | 0.13 | DDGGIVCEAOQNLAPSGHSK | 0.56 | 0.01 | 0.56 | 0.16 |
| P06905 | Proteolipid protein (myelin) | 0.35 | 0.05 | TATQISGSLCADAR | 0.30 | 0.31 | 0.37 | 0.04 |

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a SD, standard deviation from different peptides identifying the protein. In single peptide quantification, the larger standard deviation of the forward and inverse labeling experiment was taken.

b ND, not detected (d0/d4 peptide pairs were only detected in either the forward or in the reverse labeling experiments).
tional fractionation steps may lead to novel insights into the brain proteome and facilitate comparative analysis of brain compartments and the pathology of brain diseases.

CONCLUSIONS

In this study, we have described a new cysteine-tagging and complexity-reducing reagent, HysTag. Its potential as an analytical tool for protein identification and relative quantification in complex samples was demonstrated by analysis of plasma membrane fractions from two brain regions. The data were generated by an approach consisting of "on membrane" protein labeling with HysTag and digestion followed by one-dimensional chromatography of peptides. Despite the single peptide-fractionation step, 355 proteins were identified and the relative levels of 281 of these in fore- and hindbrain were determined. Implementation of an additional chromatographic separation of the peptides should enable a significant increase in the number of identified proteins. The successful application of the HysTag strategy to analysis of membrane proteins from tissue suggests that the reagent can be used for quantitative protein profiling of tissue fractions. Finally, we stress that the HysTag is easy to synthesize, allows robust and simple affinity or charge selection, and offers a convenient alternative to the original ICAT reagent.

Acknowledgments—We thank Roman Zubarev, Frank Kjeldsen, and Kim Haselmann from the Mass Spectrometry Group at University of Southern Denmark for their help in the peptide synthesis. Discussions and help by our colleagues at MDS Inc., Denmark, especially Alexandre V. Podtelejnikov, Ole Vorm, and Dan B. Kristensen, and MDS Proteomics are gratefully acknowledged.

* This work was supported by MDS Proteomics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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