Journal of proteome research

Technical Note

pubs.acs.org/jpr

Hydrophobic Interaction Chromatography for Bottom-Up Proteomics Analysis of Single Proteins and Protein Complexes

Michal Rackiewicz,[†,‡] Ludger Große-Hovest,[§] Andrew J. Alpert,[∥] Mostafa Zarei,[*,⊥] and Jörn Dengjel[*,†,‡]

[†]Department of Biology, University of Fribourg, Chemin du Musée 10, CH-1700 Fribourg, Switzerland

[‡]Department of Dermatology, Medical Center - University of Freiburg, Hauptstr. 7, D-79104 Freiburg, Germany

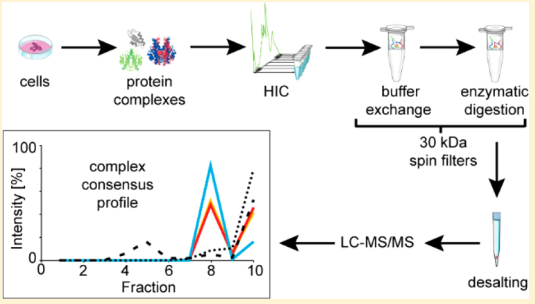
[§]SYNIMMUNE GmbH, Alte Landstrasse 42, D-72072 Tübingen, Germany

[∥]PolyLC Inc., 9151 Rumsey Road, Columbia, Maryland 21045, United States

[⊥]Solvias AG, Römerpark 2, CH-4303 Kaiseraugst, Switzerland

Supporting Information

ABSTRACT: Hydrophobic interaction chromatography (HIC) is a robust standard analytical method to purify proteins while preserving their biological activity. It is widely used to study post-translational modifications of proteins and drug−protein interactions. In the current manuscript we employed HIC to separate proteins, followed by bottom-up LC−MS/MS experiments. We used this approach to fractionate antibody species followed by comprehensive peptide mapping as well as to study protein complexes in human cells. HIC−reversed-phase chromatography (RPC)−mass spectrometry (MS) is a powerful alternative to fractionate proteins for bottom-up proteomics experiments making use of their distinct hydrophobic properties.



KEYWORDS: *antibody, protein complex, chromatography, mass spectrometry, peptide, bottom-up proteomics, enzyme, biologic*

INTRODUCTION

Knowledge of the proteome is critical for our understanding of pathophysiological processes. To study proteome alterations online liquid chromatography−tandem mass spectrometry (LC−MS/MS) is widely used. Recent technical advances allow for identification of >10 000 proteins in a cancer cell line as well as whole yeast proteome characterizations within a few hours.[1,2] To achieve such levels of proteome coverage, replicate analyses or a reduction in sample complexity, on the protein or peptide level, combined with multiple MS analyses is still critical. On the peptide level chromatography methods, like strong cation exchange (SCX) and hydrophilic interaction chromatography (HILIC), as well as high-pH reversed phase chromatography have been employed successfully.[3−6] Because of its robustness and ease of handling, the classical and still widely used approach for protein fractionation prior to LC−MS/MS is gel-based separation under denaturing conditions (SDS-PAGE).[7] Recently, size exclusion chromatography (SEC) has been employed to elucidate native protein complexes based on coelution profiles.[8−11]

For the detailed characterization of single proteins, hydrophobic interaction chromatography (HIC) has emerged as one of the key bioanalytical methods.[12] HIC is a high-resolution chromatography mode based on the interaction of weakly hydrophobic ligands of the stationary phase with hydrophobic patches on the surface of the tertiary structure of proteins.[13,14] By employment of high concentrations of structure-promoting ("kosmotropic") salts, proteins in HIC retain their conformational structure.[15] Proteins are eluted in order of increasing hydrophobicity with a gradient of decreasing ionic strength of the mobile phase. The high sensitivity of the method allows distinction between isomers and conformational states of therapeutic proteins.[16−18] Recent advances have highlighted the compatibility of HIC with direct MS analysis. Online HIC−MS as well as HIC−RPC−MS have been successfully employed for top-down proteomics analyses of single proteins and complex protein mixtures.[19,20]

We considered HIC to be a promising alternative to already established fractionation techniques for studying single proteins and protein complexes by bottom-up MS-based proteomics. The developed workflow is suitable for characterization of native soluble protein complexes as well as peptide mapping of single purified proteins and biologics.

EXPERIMENTAL SECTION

Chemicals and Reagents

All reagents were purchased from Sigma-Aldrich (Munich, Germany) unless noted otherwise.

Cell Culture

HeLa and *CaCo-2* cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Langenselbold, Germany) supplemented with 10% fetal bovine serum

Received: January 9, 2017
Published: May 9, 2017

DOI: 10.1021/acs.jproteome.7b00015
J. Proteome Res. 2017, 16, 2318−2323

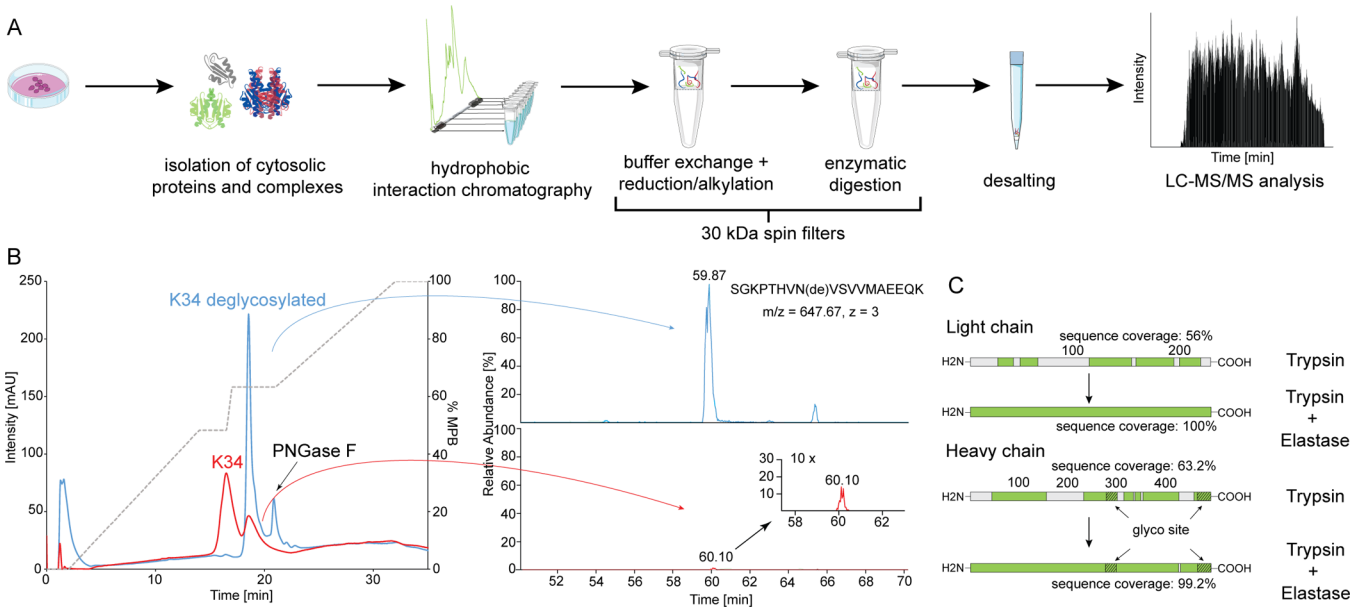


Figure 1. Workflow and analysis of antibody modifications. (A) HIC−RPC−MS workflow. Cells are lysed using a dounce homogenizer and cytosol is enriched by a centrifugation step to remove cellular membranes. Cytosol (3 mg protein) is diluted in HIC buffer A, and proteins are separated by HIC. Per 30 min HIC run, 20 fractions are collected. Each fraction is transferred to MWCO filters of 30 kDa (3 kDa in the case of antibody), and buffer is exchanged to 1% NaDOC in ABC buffer. Proteins are reduced, alkylated, and digested by trypsin. The generated peptides are filtered and desalted by STAGE tips prior to LC−MS/MS analysis. (B) Analysis of antibody modifications. The chimeric antibody K34 was resolved by HIC into two peaks (shown in red, left part of panel; HIC gradient is indicated by dotted black line). After PNGase F treatment the first peak disappeared, yielding a single antibody peak shown in blue, indicating that the first peak contained N-linked glycan moieties. Antibody fractions were processed as indicated in panel A, and the glycosylation sites were mapped to residues N298 and N456, respectively. Respective mass chromatograms for peptides containing the N456 glycosylation site are shown in the right part of the panel. The deglycosylated peptide was 100 times more abundant after PNGase F treatment. (C) Antibody sequence coverage. Whereas HIC in combination with tryptic digestion yielded ∼60% antibody sequence coverage, HIC in combination with both trypsin and elastase yielded close to 100% sequence coverage.

(Gibco, Darmstadt, Germany), 1% L-glutamine, and 1% Pen-Strep (both from PAN Biotech, Aidenbach, Germany). Cells were cultured in humidified air supplemented with 5% CO_2 at 37 °C.

Sample Preparation

Cell pellets of three 15 cm cell culture dishes were suspended in 2 mL of lysis buffer (50 mM NaOAc, 50 mM KCl (Merck, Darmstadt, Germany) with the addition of 1× protease and phosphatase inhibitors (Roche, Penzberg, Germany) and homogenized for 5 min on ice by douncing. Cell lysate was cleared of cell debris and nuclei by centrifugation at 4 °C, 3220*g* for 20 min, followed by 4 °C for 20 min at 21 000*g*. The pellet was discarded, and cell lysates were concentrated on a 100 kDa molecular weight cutoff filter (Sartorius, Göttingen, Germany). Protein concentration was determined with a BCA assay kit (Thermo Fisher, Bremen, Germany). The supernatant solution containing 3 mg of cytosolic protein complexes was diluted 1:1 (V/V) with 2 M HIC mobile phase A and used directly for HIC. Fc-optimized chimeric Igγ1 antibody K34 was deglycosylated with PNGase F at 37 °C, shaking overnight. Control K34 samples were incubated accordingly without PNGase F.

Hydrophobic Interaction Chromatography

A General Electric ÄKTA 900 HPLC system (GE Healthcare, Freiburg, Germany) equipped with a PolyPROPYL A column (3 μm, 1500 Å, 100 mm × 4.6 mm i.d.; PolyLC, Columbia, MD) was employed for HIC separation. Mobile phase A (MPA) and mobile phase B (MPB) containing either 2 M and 20 mM ammonium tartrate (AT) or the same amount of ammonium sulfate (AS), respectively, were adjusted to pH 7.0 with 10% NH_4OH. Mobile phases were filtered on a 0.22 μm vacuum filter (Corning, Corning, NY) before use.

For cytosol, a 30 min linear gradient from 100% MPA to 100% MPB, followed by 5 min of 100% MPB and 100% MPA for 5 min, was employed at a flow rate of 1 mL/min. For the K34 antibody samples the linear gradient was interrupted with two isocratic regions at 48.3% MPB (12−14.2 min) and 63.3% MPB (15−19 min). Fractions were collected one per minute throughout the gradient. Absorbance was measured at λ = 280 nm.

Filter-Based Sample Preparation for LC−MS/MS Analysis

Neighboring HIC fractions of whole cell lysates were pooled to give 10 final fractions. Samples were loaded onto 30 kDa MWCO filters (PALL, Basel, Switzerland; 3 kDa (Sartorius) for the K34 antibody) and washed with three times 500 μL of 1% sodium deoxycholate (NaDOC)/0.1 M ammonium bicarbonate (ABC). Proteins were reduced/alkylated with 5 mM tris(2-carboxyethyl)-phosphine (TCEP)/5.5 mM chloroacetamide (30 min, RT) before trypsin (Promega, Mannheim, Germany) and LysC (Wako, Neuss, Germany) digestion (each 1:100 w/w) overnight at 37 °C. Peptides were filtered and acidified with 1% trifluoroacetic acid (TFA), NaDOC was removed by centrifugation, and supernatants containing peptides were desalted by STAGE tips as described.[21] K34 antibody samples were digested with trypsin for 4 h, followed by 2 h of elastase (Promega) in ABC buffer at 37 °C. Samples were evaporated to 5 μL and acidified with 10 μL of 3% ACN/0.3% TFA (both from LGC Promochem, Wesel, Germany).

DOI: 10.1021/acs.jproteome.7b00015
J. Proteome Res. 2017, 16, 2318−2323

In-Gel Digestion

An aliquot of 350 μg protein from the cytosolic cell fraction was incubated for 10 min at 95 °C in SDS-PAGE loading buffer, reduced, and alkylated as described. Protein mixtures were separated by SDS-PAGE using 4−12% NuPAGE gels (Invitrogen, Darmstadt, Germany). Gel lanes were cut into 10 equal slices, and proteins therein were in-gel-digested with trypsin overnight at 37 °C.[7] Resulting peptides were desalted on STAGE tips.[21]

LC−MS/MS

Mass spectrometric measurements were performed on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to an Agilent 1200 nanoflow−HPLC (Agilent Technologies, Waldbronn, Germany). HPLC-column tips (fused silica) with 75 μm inner diameter (New Objective, Woburn, MA) were self-packed with Reprosil-Pur 120 ODS-3 (Dr. Maisch, Ammerbuch, Germany) to a length of 20 cm. Samples were applied directly onto the column without pre-column. A gradient of A (0.5% acetic acid (LGC Promochem) in water and B (0.5% acetic acid in 80% ACN (LC−MS grade, Wako) in water) with increasing organic proportion was used for peptide separation (loading of sample with 2% B; separation ramp: from 10−30% B within 80 min). The flow rate was 250 nL/min and for sample application 500 nL/min. The mass spectrometer was operated in the data-dependent mode and switched automatically between MS (max. of 1×10^6 ions) and MS/MS. Each MS scan was followed by a maximum of five MS/MS scans in the linear ion trap using normalized collision energy of 35% and a target value of 5′000. Parent ions with a charge state of $z = 1$ and unassigned charge states were excluded from fragmentation. The mass range for MS was m/z = 370−2000. The resolution was set to 60 000. Mass-spectrometric parameters were as follows: spray voltage 2.3 kV; no sheath and auxiliary gas flow; and ion-transfer tube temperature 200 °C.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[22] with the data set identifier PXD006217, Project Name: Hydrophobic interaction chromatography for bottom-up proteomics.

Data Analysis and Statistics

Raw MS data files were processed using MaxQuant version 1.4.1.2,[23] with a false discovery rate (FDR) < 0.01 at the level of proteins, peptides, and modifications, using default settings with the following minor changes: oxidized methionine (M) and acetylation (protein N-term) and deamidation (NQ) (only for antibody analysis) as variable modifications and carbamidomethyl (C) as fixed modification, minimum peptide length of seven amino acids, and "match between runs" (MBR) enabled with a matching time window of 1 min. Proteins and peptides were identified using a target-decoy approach with a reversed database using the Andromeda search engine integrated into the MaxQuant environment.[24] Searches were performed against the Human UniProt FASTA database (July 2015), and quantification of peptides and proteins was performed by MaxQuant. Bioinformatics analysis was performed with Multi-Experiment Viewer (http://mev.tm4.org/), Perseus, and Microsoft Excel.

RESULTS AND DISCUSSION

HIC Analysis of K34 Antibody and MS Peptide Mapping

To establish a HIC-based workflow suited for bottom-up proteomics MS analysis (Figure 1A) a set of standard proteins was used (Figure S1). We used a spin-filter-based approach to exchange HIC buffers to allow proteolytic digestion for bottom-up proteomics MS analyses.[25] Structure-promoting ammonium salts were replaced by three consecutive washing steps with 1% NaDOC in 0.1 M ammonium bicarbonate (ABC). After reduction and alkylation of cysteine side chains, proteins were digested on filter by trypsin. The resulting peptide mixtures were filtered and acidified to deplete NaDOC (see Experimental Section for details). Finally, peptides were desalted by STAGE tips and analyzed by LC−MS/MS. After achieving satisfactory results with standard proteins (data not shown), we applied our workflow to the Fc-optimized chimeric Igγ1 antibody K34.

The HIC chromatogram of the K34 antibody showed two peaks, which were not baseline-separated, at 17′ (peak I) and 19′ (peak II), indicating two distinct variants (Figure 1B, left panel, red chromatogram). The Fc-optimized chimeric antibody K34 has, in addition to the glycosylation site of human Igγ1 isotypes at N297, a second glycosylation site at N456 in the C-terminal part of its heavy chain. Indeed, after PNGase F treatment (Figure S2) only the second peak at 19 min remained, being more intense than before the treatment and indicating that peak I represented the glycosylated variant of K34 (Figure 1B, left panel, blue chromatogram). Tryptic digests from both peaks were analyzed by LC−MS/MS, yielding a protein coverage of 56% for the light chain and 63.2% for the heavy chain, respectively (Figure 1C). To unambiguously pinpoint the glycosylation sites, proteolytic digestion protocols were optimized to yield optimal sequence coverage. A consecutive proteolytic digestion protocol combing on-filter-trypsin and -elastase digestion drastically improved sequence coverage of both light (100%) and heavy chains (99.2%). Using the newly established protocol we were able to localize the glycosylation sites to N297 and N456 of the antibody heavy chain, the amino acid residues lying within N-glycosylation consensus sequence motifs N-X-S/T (with X being any amino acid except proline). The peptide containing the deglycosylated (de) N456 residue "SGKPTHVN(de)VSVVMAEEQK" showed a 100-fold increase in intensity after PNGase F treatment (Figure 1B, right panel), the peptide "EEQYN(de)-STYR" containing N297 a 200-fold increase (Figure S3). Additionally, changes in oxidation status were ruled out. Thus using HIC plus bottom-up proteomics we comprehensively characterized the antibody K34 including two glycosylation sites at N297 and N456.

HIC-based Characterization of Cytosolic Protein Complexes

Virtually all cellular processes driving cell proliferation, growth, and homeostasis depend on the dynamic regulation of physical protein−protein interactions. Thus the study of protein complexes is critical for the understanding of pathophysiological processes. Whereas protein separation by SDS-PAGE has been widely and successfully used to establish cellular protein inventories,[26] knowledge about protein interactions is lost due to the denaturing conditions during sample preparation. We used the established HIC-bottom-up proteomics workflow to analyze cytosolic protein complexes and

DOI: 10.1021/acs.jproteome.7b00015
J. Proteome Res. 2017, 16, 2318−2323

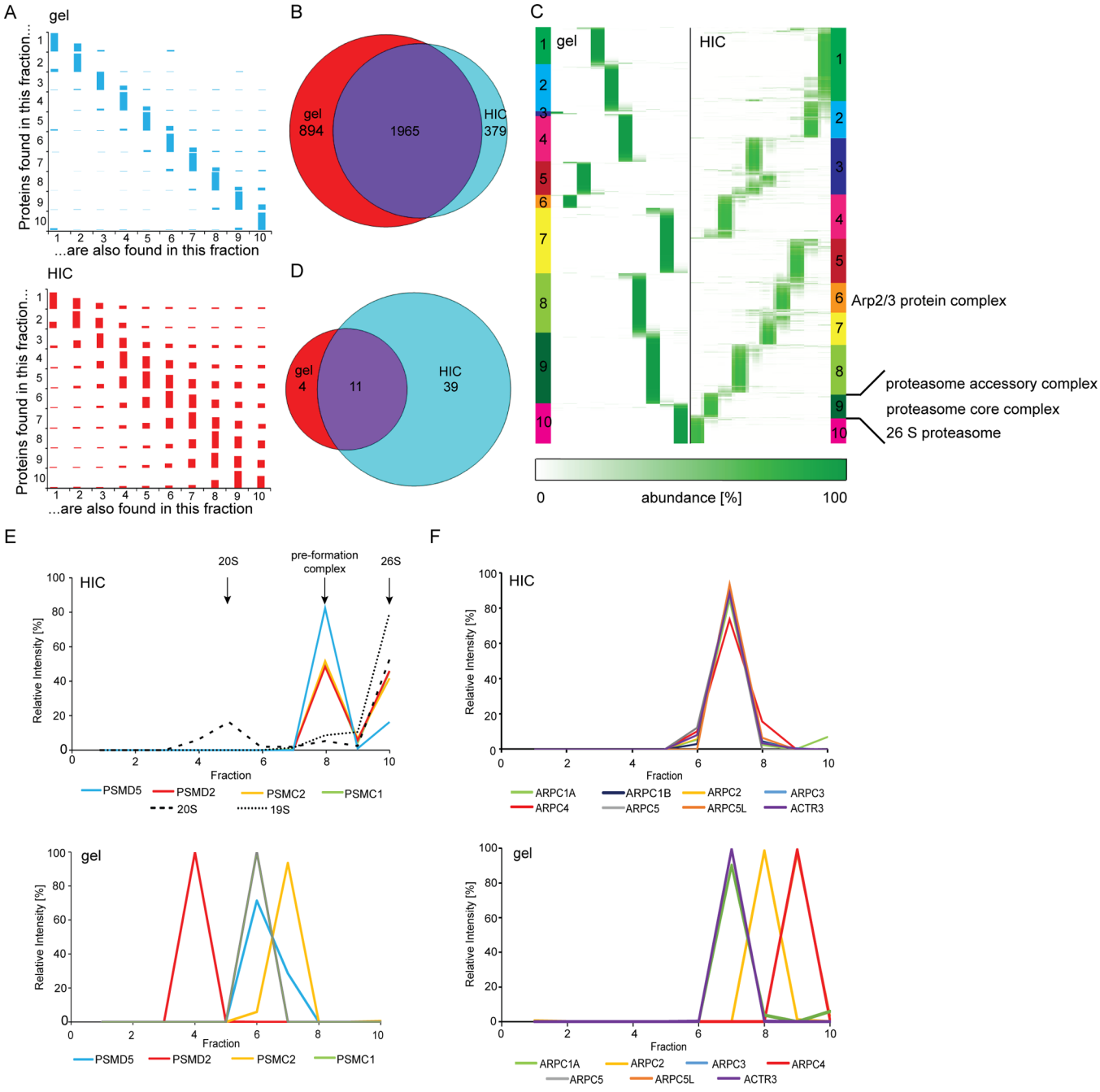


Figure 2. Analysis of protein complexes by HIC. (A) Comparison of HIC with geLC−MS. Whereas prefractionation by SDS-PAGE separated proteins according to size, HIC separated proteins according to hydrophobicity. Thus in geLC−MS proteins commonly localize to single gel fractions. By contrast, distinct protein complexes of specific proteins were found in several HIC fractions. (B) Protein identifications. Venn diagram comparing protein identifications by geLC−MS and HIC−RPC−MS. (C) Cluster analysis of protein profiles. Relative distributions of proteins fractionated by SDS-PAGE and HIC were clustered using k-means. Enrichment analysis using the CORUM annotations yielded enriched protein complex terms in HIC but not in gel clusters (see Supplemental Tables S2 and S3 for complete lists). (D) Enriched CORUM terms. Venn diagram comparing geLC−MS and HIC−RPC−MS approaches by enriched CORUM terms in respective cluster analyses shown in panel C. (E) Proteasome protein profiles. Whereas SDS-PAGE separated proteins from the proteasomal preformation complex into several fractions according to their size, HIC yielded a preformation complex consensus profile. This overlapped partially with the profiles of the 26S proteasome complexes (indicated by dotted lines). (F) Arp2/3 protein profiles. Similarly, as shown in panel E, Arp2/3 proteins eluted as a unitary complex in HIC but were separated in different fractions by SDS-PAGE. Note: geLC−MS analyses were performed with HeLa cell lysate. HIC−RPC−MS analyses were performed with both CaCo-2 cell lysate shown here and HeLa cell lysate shown in Figure S4.

compared the generated data to classical gel-based bottom-up proteomics analyses.

Whereas the separation by size in SDS-PAGE leads to the focusing of proteins into single gel bands, HIC leads to a broader distribution of proteins (Figure 2A). More than 66% of proteins are found in single bands in SDS-PAGE, but only 24% of proteins elute in single HIC fractions. This broader distribution is very likely due to participation of proteins in multiple complexes (see below). The broader distribution and thus less relative concentration of proteins may also be the

DOI: 10.1021/acs.jproteome.7b00015
J. Proteome Res. 2017, 16, 2318−2323

reason for lower identification rates in HIC−MS compared with SDS-PAGE-MS (Figure 2B and Supplemental Table S1). Critically, separation by HIC is robust and cell-line-independent. Cytosol fractionations of HeLa and CaCo-2 cell samples gave very similar results (Figure 2 and Figure S4).

To elucidate the suitability of HIC for the analysis of protein complexes by bottom-up MS-based proteomics, we clustered elution profiles and performed bioinformatics enrichments analyses on respective clusters using the CORUM annotation of protein complexes (Figure 2C).[27] As anticipated, we identified significantly more enriched complexes in HIC compared with SDS-PAGE analyses (Figure 2D, $p < 0.05$, BH-corrected; Supplemental Tables S2 and S3), two examples being the proteasome and the Arp2/3 protein complex. Proteins PSMC1 and PSMC2 together with PSMD2 and PSMD5 are known to associate in a transient preformation complex of the 26S proteasome.[28,29] In SDS-PAGE, proteins distributed in several gel bands according to their size (Figure 2E). In HIC two peaks containing all of the proteins were observed: The second peak eluted together with other members of the PSMA, PSMB, PSMC, and PSMD protein families, indicating that this peak corresponds to the 26S proteasome. Thus HIC was able to separate the 20S, 26S proteasome and the preformation complex. Also, the Arp2/3 complex eluted as a unit in HIC, whereas its constituents were separated by size in SDS-PAGE (Figure 2F).

CONCLUSIONS

Taken together, HIC in combination with bottom-up proteomics allows peptide mapping and detailed post-translational modification (PTM) analysis of single proteins as well as the study of protein complexes and interactions. Because the mode of separation is based on hydrophobicity and not on size, as per SDS-PAGE and SEC, this approach will be particularly useful for studying the role of PTMs in the formation of complexes as well as drug−protein interactions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00015.

Figure S1: HIC analyses of standard proteins. Figure S2: HIC analyses of PNGase F. Figure S3: Mass chromatograms of K34 antibody peptides containing the N298 glycosylation site. Figure S4: HIC−RPC−MS analyses of HeLa cell cytosol. (PDF)

Supplemental Table S1: Protein identifications by SDS-PAGE and HIC. (XLSX)

Supplemental Table S2: Enriched CORUM terms from HIC analysis. (XLSX)

Supplemental Table S3: Enriched CORUM terms from SDS-PAGE analysis. (XLSX)

AUTHOR INFORMATION

Corresponding Authors

*M.Z.: E-mail: Mostafa.Zarei@SOLVIAS.com. Tel: +41 61 845 6563.

*J.D.: E-mail: joern.dengjel@unifr.ch. Tel: +41 26 300 8631. Fax: +41 26 300 9741.

ORCID

Jörn Dengjel: 0000-0002-9453-4614

Notes

The authors declare the following competing financial interest(s): A.J.A. works for the company that supplied the HIC column.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[22] with the data set identifier PXD006217, Project Name: Hydrophobic interaction chromatography for bottom-up proteomics.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the Swiss National Science Foundation, grant 31003A-166482/1 (J.D.).

REFERENCES

(1) Hebert, A. S.; Richards, A. L.; Bailey, D. J.; Ulbrich, A.; Coughlin, E. E.; Westphall, M. S.; Coon, J. J. The one hour yeast proteome. *Mol. Cell. Proteomics* **2014**, *13* (1), 339−47.

(2) Nagaraj, N.; Wisniewski, J. R.; Geiger, T.; Cox, J.; Kircher, M.; Kelso, J.; Paabo, S.; Mann, M. Deep proteome and transcriptome mapping of a human cancer cell line. *Mol. Syst. Biol.* **2011**, *7*, 548.

(3) Batth, T. S.; Olsen, J. V. Offline High pH Reversed-Phase Peptide Fractionation for Deep Phosphoproteome Coverage. *Methods Mol. Biol.* **2016**, *1355*, 179−92.

(4) Chen, W.; Wang, S.; Adhikari, S.; Deng, Z.; Wang, L.; Chen, L.; Ke, M.; Yang, P.; Tian, R. Simple and Integrated Spintip-Based Technology Applied for Deep Proteome Profiling. *Anal. Chem.* **2016**, *88* (9), 4864−71.

(5) Zarei, M.; Sprenger, A.; Rackiewicz, M.; Dengjel, J. Fast and easy phosphopeptide fractionation by combinatorial ERLIC-SCX solid-phase extraction for in-depth phosphoproteome analysis. *Nat. Protoc.* **2015**, *11* (1), 37−45.

(6) Zhou, H.; Di Palma, S.; Preisinger, C.; Peng, M.; Polat, A. N.; Heck, A. J.; Mohammed, S. Toward a comprehensive characterization of a human cancer cell phosphoproteome. *J. Proteome Res.* **2013**, *12* (1), 260−71.

(7) Shevchenko, A.; Tomas, H.; Havlis, J.; Olsen, J. V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc* **2007**, *1* (6), 2856−60.

(8) Kristensen, A. R.; Gsponer, J.; Foster, L. J. A high-throughput approach for measuring temporal changes in the interactome. *Nat. Methods* **2012**, *9* (9), 907−9.

(9) Park, S. J.; Ahn, H. S.; Kim, J. S.; Lee, C. Evaluation of Multi-tRNA Synthetase Complex by Multiple Reaction Monitoring Mass Spectrometry Coupled with Size Exclusion Chromatography. *PLoS One* **2015**, *10* (11), e0142253.

(10) Wan, C.; Borgeson, B.; Phanse, S.; Tu, F.; Drew, K.; Clark, G.; Xiong, X.; Kagan, O.; Kwan, J.; Bezginov, A.; Chessman, K.; Pal, S.; Cromar, G.; Papoulas, O.; Ni, Z.; Boutz, D. R.; Stoilova, S.; Havugimana, P. C.; Guo, X.; Malty, R. H.; Sarov, M.; Greenblatt, J.; Babu, M.; Derry, W. B.; Tillier, E. R.; Wallingford, J. B.; Parkinson, J.; Marcotte, E. M.; Emili, A. Panorama of ancient metazoan macromolecular complexes. *Nature* **2015**, *525* (7569), 339−44.

(11) Diedrich, B.; Rigbolt, K. T.; Roring, M.; Herr, R.; Kaeser-Pebernard, S.; Gretzmeier, C.; Murphy, R. F.; Brummer, T.; Dengjel, J. Discrete cytosolic macromolecular BRAF complexes exhibit distinct activities and composition. *EMBO J.* **2017**, *36* (5), 646−63.

(12) O'Connor, B. F.; Cummins, P. M. Hydrophobic Interaction Chromatography. *Methods Mol. Biol.* **2017**, *1485*, 355−63.

(13) Hjertén, S. Some general aspects of hydrophobic interaction chromatography. *Journal of Chromatography A* **1973**, *87* (2), 325−31.

(14) Hjertén, S.; Rosengren, J.; Påhlman, S. Hydrophobic interaction chromatography. *Journal of Chromatography A* **1974**, *101* (2), 281−8.

DOI: 10.1021/acs.jproteome.7b00015
J. Proteome Res. 2017, 16, 2318−2323

(15) Alpert, A. J. High-performance hydrophobic-interaction chromatography of proteins on a series of poly(alkyl aspart-amide)-silicas. *Journal of Chromatography A* **1986**, *359*, 85−97.
(16) Boyd, D.; Kaschak, T.; Yan, B. HIC resolution of an IgG1 with an oxidized Trp in a complementarity determining region. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879* (13−14), 955−60.
(17) Grebenau, R. C.; Goldenberg, D. M.; Chien-Hsing, C.; Koch, G. A.; Gold, D. V.; Kunz, A.; Hansen, H. J. Microheterogeneity of a purified IgG1 due to asymmetric Fab glycosylation. *Mol. Immunol.* **1992**, *29* (6), 751−8.
(18) Wakankar, A. A.; Borchardt, R. T.; Eigenbrot, C.; Shia, S.; Wang, Y. J.; Shire, S. J.; Liu, J. L. Aspartate isomerization in the complementarity-determining regions of two closely related monoclonal antibodies. *Biochemistry* **2007**, *46* (6), 1534−44.
(19) Chen, B.; Peng, Y.; Valeja, S. G.; Xiu, L.; Alpert, A. J.; Ge, Y. Online Hydrophobic Interaction Chromatography-Mass Spectrometry for Top-Down Proteomics. *Anal. Chem.* **2016**, *88* (3), 1885−91.
(20) Xiu, L.; Valeja, S. G.; Alpert, A. J.; Jin, S.; Ge, Y. Effective protein separation by coupling hydrophobic interaction and reverse phase chromatography for top-down proteomics. *Anal. Chem.* **2014**, *86* (15), 7899−906.
(21) Rappsilber, J.; Mann, M.; Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* **2007**, *2* (8), 1896−906.
(22) Vizcaino, J. A.; Csordas, A.; del-Toro, N.; Dianes, J. A.; Griss, J.; Lavidas, I.; Mayer, G.; Perez-Riverol, Y.; Reisinger, F.; Ternent, T.; Xu, Q. W.; Wang, R.; Hermjakob, H. 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* **2016**, *44* (D1), D447−56.
(23) Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **2008**, *26* (12), 1367−72.
(24) Cox, J.; Neuhauser, N.; Michalski, A.; Scheltema, R. A.; Olsen, J. V.; Mann, M. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* **2011**, *10* (4), 1794−805.
(25) Wisniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **2009**, *6* (5), 359−62.
(26) Sprenger, A.; Weber, S.; Zarai, M.; Engelke, R.; Nascimento, J. M.; Gretzmeier, C.; Hilpert, M.; Boerries, M.; Has, C.; Busch, H.; Bruckner-Tuderman, L.; Dengjel, J. Consistency of the proteome in primary human keratinocytes with respect to gender, age, and skin localization. *Mol. Cell. Proteomics* **2013**, *12* (9), 2509−21.
(27) Ruepp, A.; Waegele, B.; Lechner, M.; Brauner, B.; Dunger-Kaltenbach, I.; Fobo, G.; Frishman, G.; Montrone, C.; Mewes, H. W. CORUM: the comprehensive resource of mammalian protein complexes–2009. *Nucleic Acids Res.* **2010**, *38* (Database issue), D497−501.
(28) Kaneko, T.; Hamazaki, J.; Iemura, S.; Sasaki, K.; Furuyama, K.; Natsume, T.; Tanaka, K.; Murata, S. Assembly pathway of the Mammalian proteasome base subcomplex is mediated by multiple specific chaperones. *Cell* **2009**, *137* (5), 914−25.
(29) Roelofs, J.; Park, S.; Haas, W.; Tian, G.; McAllister, F. E.; Huo, Y.; Lee, B. H.; Zhang, F.; Shi, Y.; Gygi, S. P.; Finley, D. Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature* **2009**, *459* (7248), 861−5.

DOI: 10.1021/acs.jproteome.7b00015
J. Proteome Res. 2017, 16, 2318−2323