Targeting Peptide Termini, a Novel Immunoaffinity Approach to Reduce Complexity in Mass Spectrometric Protein Identification

Sibylle Hoepepe, Thomas D. Schreiber, Hannes Planatscher, Andreas Zell, Markus F. Templin, Dieter Stoll, Thomas O. Joos, and Oliver Poetz

Mass spectrometry and peptide-centric approaches are powerful techniques for the identification of differentially expressed proteins. Despite enormous improvements in MS technologies, sample preparation and efficient fractionation of target analytes are still major bottlenecks in MS-based protein analysis. The complexity of tryptically digested whole proteomes needs to be considerably reduced before low abundance proteins can be effectively analyzed using MS/MS. Sample preparation strategies that use peptide-specific antibodies are able to reduce the complexity of tryptic digests and lead to a substantial increase in throughput and sensitivity; however, the number of peptide-specific capture reagents is low, and consequently immunoaffinity-based approaches are only capable of detecting small sets of protein-derived peptides. In this proof-of-principle study, special anti-peptide antibodies were used to enrich peptides from a complex mixture. These antibodies recognize short amino acid sequences that are found directly at the termini of the peptides. The recognized epitopes consist of three or four amino acids only and include the terminally charged group of the peptide. Because of its limited length, antibodies recognizing the epitope will enrich not only one peptide but a whole class of peptides that share this terminal epitope. In this study, β-catenin-derived peptides were used to demonstrate that it is possible (i) to effectively generate antibodies that recognize short C-terminal peptide epitopes and (ii) to enrich and identify peptide classes from a complex mixture using these antibodies in an immunoaffinity MS approach. The expected β-catenin peptides and a set of 38 epitope-containing peptides were identified from trypsin-digested cell lysates. This might be a first step in the development of proteomics applications that are based on the use of peptide class-specific antibodies. Molecular & Cellular Proteomics 10: 1–11, 2011.

Mass spectrometry (MS) is the key technology in proteomics research. Unbiased, proteomics discovery approaches require extensive sample processing to reduce sample complexity prior to MS analysis. The proteins in a biological sample therefore must be separated using physicochemical methods, e.g. two-dimensional gels. Alternatively, proteins can be enzymatically fragmented, and the resulting peptides can be separated using multidimensional chromatography (multi-dimensional protein identification technology) (1–3). These technologies are well established and allow the identification of a large number of proteins present in a sample (>1000). Up to 100 differentially expressed proteins can be identified in one experiment (4). However, it is necessary to validate the identified proteins, i.e. biomarker candidates, using much larger sample cohorts. For validation, the analytical strategy has to be switched from a knowledge-independent to a knowledge-based approach because of cost and time restrictions. Multiple reaction monitoring (5, 6) and affinity-based methods (7, 8) lead to an increase in throughput and sensitivity. Peptide-specific antibodies proved to be a valuable tool to capture signature peptides derived from the potential biomarkers (9, 10). The target analytes can be isolated from tryptically digested biological samples in a single affinity step, which enables the absolute quantification of selected peptides from digested clinical samples to physiologically relevant analyte concentrations (ng/ml) with a high level of precision (<10% coefficient of variation) and accuracy. For example, Whiteaker et al. (11) generated peptide-specific antibodies that enabled the capture of nine signature peptides from plasma samples in a multiplexed fashion. Because this method relies on one specific antibody per target, several tens of thousands of antibodies need to be generated for applications in large scale proteomics research (12). However, once the antibodies have been generated and validated, immunoaffinity procedures are a rapid, highly selective means of enriching material for further analysis.

Immunoaffinity-based methods are also used in unbiased approaches to reduce sample complexity. Antibodies that are able to bind generically to phosphorylated tyrosine or acetylated lysine residues were used for the enrichment of tryptic peptide fragments derived from post-translationally modified proteins.
Targeting Peptide Termini

![Diagram showing Affinity Step and Detection](image)

Fig. 1. Immunoaffinity MS involving use of peptide-specific antibodies versus use of TXP antibodies. Compared with classical peptide-specific antibodies with one-analyte specificity, TXP antibodies are generated with targeted multispecificity capable of capturing peptide subsets that share the same terminus (approach shaded in gray).

proteins (13–17). A consequent extension of existing immunoaffinity MS concepts is the development and use of peptide group-specific antibodies targeting common peptide sequences. The use of those antibodies for enrichment strategies in global proteomics approaches was recently introduced by us and other scientists (18, 19). In the triple X proteomics (TXP), concept (18), we use antibodies that are capable of trapping groups of peptides that share a common epitope of three to four amino acids at the N- or C-terminal end; this short amino acid sequences. The use of those antibodies for enrichment strategies in global proteomics approaches was recently introduced by us and other scientists (18, 19). In the triple X proteomics (TXP), concept (18), we use antibodies that are capable of trapping groups of peptides that share a common epitope of three to four amino acids at the N- or C-terminal end; this short amino acid sequence is referred to as TXP epitope. In contrast to classical peptide-specific antibodies, these antibodies can be selected and generated to bind dozens to hundreds of peptides that share the same epitope (Fig. 1).

The present study validates the TXP concept using β-catenin as a target analyte. Two polyclonal rabbit antibodies were generated against two short peptide termini, LGYR and AMTR, that are present in two theoretical β-catenin-specific signature peptides. Affinity-purified antibodies were thoroughly characterized using positional scanning peptide libraries in combination with suspension bead array technology. Using this procedure, amino acid residues of the epitope relevant for antibody-antigen interaction could be determined (20). Furthermore, positional scanning peptide libraries were applied in a peptide capture assay with MS readout to determine cross-reactivities of the antibodies. Finally, the characterized affinity-purified antibodies were applied in an immunoaffinity enrichment step of peptide classes from a tryptically digested cell line. The subsequent MALDI MS/MS readout permitted the direct identification of β-catenin and other signature peptides in these complex biological samples. This proof-of-principle study reveals the potential of the TXP concept in providing antibodies for knowledge-dependent and knowledge-independent protein analysis.

### EXPERIMENTAL PROCEDURES

#### Polyclonal TXP Antibody Generation

Antigens were generated by conjugating the peptides to keyhole limpet hemocyanin (KLH). KLH (Pierce) was reconstituted in sterile double distilled H2O to a concentration of 10 mg/ml. Sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide ester (sMBS; Pierce) was freshly dissolved in DMSO at a concentration of 40 mg/ml. 1 volume of sMBS solution was mixed with 10 volumes of KLH solution and incubated at room temperature (RT) for 1 h on an orbital shaker. Any sMBS that had not reacted was removed by size exclusion chromatography using a HiTrap desalting column (GE Healthcare) on an AKTA FPLC chromatography system (GE Healthcare). Cysteine-containing peptide antigens were dissolved in PBS at a concentration of 4 mg/ml. The peptides were reduced with 1 eq of tris(2-carboxyethyl)phosphine (Sigma-Aldrich) prior to conjugation to ensure that the thiol groups of the peptides were not oxidized. The peptides were mixed 1:1 (w/w) with the preactivated KLH and gently mixed for 3 h at RT. Rabbits were immunized with these conjugates at Pineda Antibody Service, Berlin, Germany, and sacrificed after day 81 to obtain polyclonal serum. The peptides were purchased from Intavis, Reutlingen, Germany.

#### Affinity Purification of Polyclonal Antibodies

The polyclonal antibodies were affinity-purified using peptide-loaded BSA that was covalently coupled to preactivated Sepharose columns. Preactivated BSA (2 mg, 2 mg/ml; Pierce) was dissolved according to the manufacturer’s instructions. 400 µl of reduced peptide (5 mg/ml) containing a cysteine for immobilization, a Myc tag sequence as a spacer, and the targeted epitope, LGYR or AMTR, sequence was added and incubated for 2 h at RT. Uncoupled peptides were removed using a Microcon centrifugal filter device (Millipore, Billerica, MA; cutoff molecular mass, 50,000 Da). A HiTrap HP 1-m1 column (GE Healthcare) was prepared as specified by the manufacturer using 1 mg of peptide conjugate. Polyclonal antibodies were immunopurified using an FPLC chromatography system (GE Healthcare) according to a standard antibody purification protocol.

#### Epitope Library Scanning

Antibody Binding Assay—A two-step procedure using standard 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysuccinimide chemistry according to Kurzeder et al. (21) was used for the covalent coupling of peptides to carboxylated fluorescent microspheres (Luminex Corp., Austin, TX) for the generation of bead-based peptide arrays. Peptides used for coupling consisted of an N-terminal cysteine followed by the spacer sequence HSG, the epitope sequences, or variations of the epitope sequences LGYR and AMTR. The epitope sequences LGYR and AMTR were synthesized as peptide amides to assess the C terminus specificity of the antibodies. Additionally, positional scanning peptide libraries were synthesized and coupled in which one amino acid of the epitope sequence was replaced by a random amino acid of the pool of 19 proteinogenic amino acids and aminobutyric acid (replacing cysteine). Peptides

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1 The abbreviations used are: TXP, triple X proteomics; IP, immunoprecipitation; KLH, keyhole limpet hemocyanin; SISCAPA, stable isotope standards and capture by anti-peptide antibodies; sMBS, sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide ester; NOG, n-octyl β-D-glucopyranoside; PAC, prespotted anchor chip; PEEK, polyetheretherketone; S/N, signal-to-noise; GPMDB, Global Proteome Machine Database.
were purchased from Intavis. A bead mixture for the multiplex analysis of purified antibodies was generated by pooling equal amounts of peptide-coupled microspheres. The quantity of each bead type was adjusted to 100 beads/μL. The microsphere mixture was transferred into 96-well filter plates (800 microspheres of each bead type/well), mixed with 30 μL of purified antibody (0.04 μg/mL), and incubated for 1 h at RT in an Eppendorf Thermomixer at 650 rpm. The microspheres were subsequently washed twice with 100 μL of PBS. 30 μL of phycoerythrin-labeled detection antibody (2.5 μg/mL; Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated for 30 min at RT and 650 rpm. The bead type-specific fluorescence was determined with a LumineX 100 IS system.

Peptide Capture Assay—To evaluate capture specificity positional scanning libraries of AMTR and LGYR, peptides were incubated with anti-AMTR and anti-LGYR antibodies. 10 pmol of a peptide library was incubated with 1 μg of antibody for 1 h in PBS, 0.3% n-octyl β-D-glucopyranoside (N OG; Sigma-Aldrich) followed by 1-h incubation with 5 μL of Dynabeads Protein G (Invitrogen). Washing steps were performed twice with PBS, 0.3% N OG and three times with 50 mM ammonium hydrogen carbonate buffer (pH 7.4), 0.3% N OG. Elution was performed with 20 μL of 1% formic acid. 1 μL of eluate was spotted onto a disposable prespotted anchor chip (PAC; Bruker Daltonics, Bremen, Germany), and peaks between 600 and 1000 Da were analyzed with an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). The spectrometer was calibrated using prespotted calibrants on the C 18 column (OPTI-GUARD Biocompatible, 1 mm; Optimize Technologies, Oregon City, OR). The trapping column was washed with an Easy-nLC system (Proxeon A/S, Odense, Denmark) at 2 μL/min with 5 column volumes of 0.1% trifluoroacetic acid (TFA) in water to remove the salt. The trapped fragments were eluted with 8 column volumes of 80% acetonitrile (ACN), 0.1% TFA in water at 2 μL/min. The eluate was spotted onto a prespotted MALDI target (PAC; Bruker Daltonics). Dried samples were desalted by short dipping of the target into 10 μL monobasic ammonium phosphate, 0.1% TFA.

Preparation of Antibody Affinity Columns

Immunoaffinity material was prepared according to the protocol published by Anderson et al. (22) using POROS G 20 beads (Applied Biosystems, Foster City, CA) and purified anti-AMTR and anti-LGYR antibodies. Affinity columns were prepared in 10 × 4.0-mm PEEK columns with 2-μm PEEK frits and polytetrafluoroethylene sealing gaskets (Alltech Grom GmbH, Rottenburg, Germany) using a syringe pump.

Cell Treatment and Lysis

Cultured HEK293 cells (ATCC, Manassas, VA) were treated for 6 h either with 20 μM GSK3 inhibitor SB216763 (Sigma-Aldrich) or with DMSO as a solvent control. After treatment, cells were washed twice with PBS and harvested using a rubber policeman. The cell suspension was adjusted to 100 beads/mL. Solutions were shaken at 450 rpm at 60 °C for 1 h. 20 mM iodoacetamide was then added, and the solutions were incubated in the dark at RT for 30 min. Trypsin (Promega, Madison, WI) was added to the lysate at an enzyme/substrate ratio of 1:40 (w/w), and the mixture was incubated at 450 rpm overnight at 37 °C. Trypsin activity was stopped by the addition of 1 μM protease inhibitor (phenylmethylsulfonyl fluoride; Sigma-Aldrich). Proteolytic digestion was assessed by SDS-PAGE prior to further analyses.

Sample Digestion

Samples were digested using trypsin. Protein extracts were reduced by adding dithiothreitol (DTT) to a concentration of 5 mM. Solutions were shaken at 450 rpm at 60 °C for 1 h. 20 mM iodoacetamide was then added, and the solutions were incubated in the dark at RT for 30 min. Trypsin (Promega, Madison, WI) was added to the lysate at an enzyme/substrate ratio of 1:40 (w/w), and the mixture was incubated at 450 rpm overnight at 37 °C. Trypsin activity was stopped by the addition of 1 μM protease inhibitor (phenylmethylsulfonyl fluoride; Sigma-Aldrich). Proteolytic digestion was assessed by SDS-PAGE prior to further analyses.

Affinity MS Procedure

An ÄKTA FPLC system (GE Healthcare) was used for immunoaffinity chromatography. The different antibody affinity columns described above were installed sequentially in a row and washed with PBS. 50 μL of digested HEK293 lysate in 2 mL of PBS was applied to the columns at 100 μL/min. Unbound peptides were washed with 7 column volumes of PBS at 100 μL/min. After separating the columns, the antibody-bound peptides were eluted with 14 column volumes of 100 mM citrate buffer, pH 2.5 at 1 mL/min. The eluate was trapped onto a C 18 column (OPTI-GUARD Biocompatible, 1 mm; Optimize Technologies, Oregon City, OR). The trapping column was washed with an Easy-nLC system (Proxeon A/S, Odense, Denmark) at 2 μL/min with 5 column volumes of 0.1% trifluoroacetic acid (TFA) in water to remove the salt. The trapped fragments were eluted with 8 column volumes of 80% acetonitrile (ACN), 0.1% TFA in water at 2 μL/min. The eluate was spotted onto a prespotted MALDI target (PAC; Bruker Daltonics). Dried samples were desalted by short dipping of the target into 10 μL monobasic ammonium phosphate, 0.1% TFA.

Protein Identification by MALDI-TOF MS/MS

MS and MS/MS spectra were obtained using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) controlled by the flexControl 3.0 software package. All experiments were carried out in positive ion reflectron mode with a deflector cutoff up to 500 Da. All spectra were analyzed using the flexAnalysis 3.0 software (Bruker Daltonics). The spectrometer was calibrated using prespotted calibrants on the PAC target and insulin (Sigma-Aldrich). MS masses were automatically annotated with the flexAnalysis peptide mass fingerprint method, which resulted in a peak list in a mass range between 700 and 5000 Da. Only peaks with a signal-to-noise (S/N) ratio greater than 10 were chosen for MS/MS analysis. The selected peaks were fragmented using the LIFT™ technology to identify the peptides. The laser power and number of shots (3000–4000) were adjusted by the operator. Monoisotopic masses of annotated peaks were compared with Swiss-Prot database entries (version 57.13; containing 514,212 sequence entries) using the BioTools 3.1 software (Bruker Daltonics) and the Mascot 2.2 search engine (Matrix Science, London, UK). Mascot searches were restricted to human taxonomy and trypsin digestion without partial cleavage. Variable modifications such as acetylation; deamination of asparagine or glutamine; carbamidomethylation of cysteine; oxidation of methionine; phosphorylation of serine, threonine, and tyrosine; and the cyclization of glutamine were taken into account. Mass tolerances were 90 ppm for precursor ions and 1.3 Da for fragment ions. Only peptides that were identified by Mascot ion scores that indicated identity or extensive homology with p < 0.05 were regarded as valid. The data associated with this study may be downloaded from ProteomeCommons.org. Tranche using the following hash: 92v9oUt47UtqLJbY89C753Pdmggx2QR/ fileSZA6ibvl16hi7wek1R+5eyFWzr6hsSaQzg8G6gMbSjppqNp52Ra/X1cAAAADAFA99A=/=

RESULTS

Triple X Proteomics Concept—The use of peptide-specific antibodies that recognize peptide fragments generated by the enzymatic digestion of a proteome is an effective method for enriching target peptides from highly complex samples. Combined with MS analysis, it is a valuable tool for targeted proteomics. Instead of the immunoprecipitation of a single peptide, the method described here uses antibodies for the enrichment of peptides that share the same terminal amino acid sequence, the TXP epitope. Thereby, each TXP antibody
is multispecific. After enrichment, the peptide classes can be elucidated by MS and MS/MS readout. If the peptide signal turns out to be unique, the peptides can be mapped back to their original proteins. TXP antibodies can be generated from the epitopes of different signature peptides of proteins of interest capable of simultaneously enriching several signature peptides. This is a unique possibility for reducing and optimizing the number of antibodies required for immunoaffinity enrichment on a proteome-wide scale.

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\[ \text{\(\beta\)-Catenin, which plays a key role in the WNT pathway and tumorigenesis (23–25), was chosen as a model molecule to prove the feasibility of the TXP concept. Two signature peptides were selected (GNPEEEDVDTSQVLYEWEQGFSQSFTQEQQAVIDADGQYAMTR and TEPMAWNETADLGDIAGQGEALGyr), and polyclonal antibodies were raised in rabbits targeting the two C termini, AMTR and LGYR.} \]}

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\[ Characterization of TXP Antibody Epitope Using Peptide Arrays and Antibody Binding Assay—The TXP approach requires antibodies that are directed against short terminal epitopes to enrich peptide classes according to their common terminal sequences, the TXP epitopes. This calls for a detailed characterization of the antibody binding activities. The selectivity of the TXP epitope antibody needs to be assessed prior to applying the antibody in a bioanalytical workflow. The assessment of antibody specificity for a certain peptide can be done with arrays containing small peptide libraries (20, 26). To characterize the affinity-purified polyclonal TXP antibodies, suspension bead arrays were designed that contained positional scanning peptide libraries of the targeted sequences and amidated versions to demonstrate that only the free terminal end is recognized by the TXP antibodies.} \]
To evaluate the terminus selectivity, the binding of the purified anti-AMTR and anti-LGYR antibodies to peptides containing the C-terminal AMTR or LGYR sequence was compared with peptides with an amidated C terminus, mimicking a target sequence that occurs within a peptide. The results presented in Fig. 2A show that for both anti-AMTR (left) and anti-LGYR antibodies (right) less than 10% signal was obtained for the antibody interaction with the amidated peptides in reference to the target sequence with a carboxyl function at the terminus. This indicates that the carboxyl function of the C terminus of the AMTR or LGYR sequence is a substantial part of the epitope and that the antibody is terminus-selective. Therefore, the antibodies were not expected to bind to the internal peptide sequences AMTR and LGYR if they occur within non-cleaved or non-cleavable sequences.

Next, epitope specificities of the purified antibodies were investigated with peptide arrays that contained positional scanning peptide libraries of the TXP epitopes. These assays allowed the detailed analysis of the relevance of a particular amino acid within the epitope for antigen-antibody interaction. Four different positional scanning peptide libraries were synthesized for each target sequence. In each library, one individual amino acid position was randomized by including any one of 19 amino acids or butyric acid (as a replacement for cysteine). Specificity of the antibody for a certain amino acid at a certain position was indicated by the loss of signal on a peptide bead made from a positional scanning library of this kind compared with signals obtained from interaction with the targeted epitope (20). Fig. 2A illustrates the results obtained for the purified anti-AMTR and anti-LGYR antibodies. Referring to the anti-AMTR antibodies, replacing the amino acid alanine, threonine, or arginine decreased the signal to less than 30% compared with the signal of the targeted epitope. This indicates a high specificity for these three positions. However, replacing methionine had a far less significant effect on antigen-antibody binding. Thus, the amino acids Ala, Thr, and Arg contribute most to the interaction with the antibody. An AXTR binding motif was identified for the anti-AMTR antibodies.

Use of the four LGYR libraries showed that the replacement of the amino acids leucine, glycine, and tyrosine reduced the binding of the anti-LGYR antibody by 50%, whereas the LGYX library showed a decrease of 60%. This indicates that all amino acids in the TXP epitope contribute fairly equally to antibody binding.

Characterization of TXP Antibody Epitope Using Positional Scanning Peptide Libraries in Peptide Capture Assay—Peptide arrays provided detailed information about the antibody binding characteristics in terms of the distinct amino acid and their positions within the epitope. However, in the sought-after application, immunoaffinity enrichment, the conditions are different from the solid phase antigen-antibody binding assay described above. Here, the capture capabilities and a detailed knowledge of the cross-reactivity of the antibodies are more important than their binding properties; therefore, the specificity of the two TXP antibodies was characterized in detail using a peptide capture assay. Positional scanning peptide libraries of AMTR and LGYR were generated and used for immunoprecipitation either with an anti-AMTR or anti-LGYR antibody. MALDI-TOF readout of the precipitates allowed the relative degree of enrichment by immunoprecipitation to be estimated for a specific peptide normalized to the targeted AMTR or LGYR peptide. The normalization to the targeted peptide signal enabled the comparison of the signals despite different MALDI ionization probabilities inherent to the peptides. Thus, if the same quantity of a peptide as the standard peptide was captured by the antibody, the ratio given in Fig. 2B would not change before immunoprecipitation (IP) and after IP. It was not always possible to identify the respective peptide with asparagine as a replacement as the peak appeared within the isotope cluster of leucine/isoleucine due to the mass difference of only 1 Da.

Using the affinity-purified anti-AMTR antibodies in the peptide capture assay, it turned out that methionine in the epitope can be replaced by most other amino acids. The analysis of the immunoprecipitates of the XMT, AMXR, or AMTX library revealed only low cross-reactivities of the antibody to other amino acids placed at this position (Fig. 2B). In general, immunoprecipitated peptides from the XMT and AMXR libraries contained amino acids with similar structural properties as the replaced alanine and threonine, i.e., small amino acids or small hydroxyl group-containing amino acids. Nevertheless, all ratios for the non AMTR sequence-containing captured peptides were lower after IP compared with the ratios before IP, indicating a decrease of the respective peptide ratio after elution and hence a selective enrichment of peptides with the targeted TXP epitope AMTR.

The affinity-purified anti-LGYR antibodies captured some peptides containing amino acid replacements for the amino acids leucine and tyrosine in the targeted LGYR epitope. Peptides with an α-aminobutyric acid or methionine instead of leucine appeared with a higher peak area after IP, suggesting an enrichment by IP. All other amino acid replacements resulted in a decrease of the respective peptide with regard to the LGYR sequence. Although the number of amino acids that are able to replace glycine is relatively high, most of them appear with peak area ratios of ½ or even lower when compared with the LGYR sequence. This implies that the anti-LGYR antibodies strongly enrich their targeted TXP epitope from the library peptides.

Thus, the results of the suspension bead array-based antibody binding assay shown in Fig. 2A are in line with the findings of the MS-based readout of the peptide capture assay (Fig. 2B). For anti-AMTR antibodies, the chance of capturing peptides containing amino acids other than methionine in the C-terminal AMTR sequence is rather high. The results for the anti-LGYR antibodies argue for a good specificity for all amino acids in the LGYR epitope. The selectivity of both antibodies for arginine at the C terminus.
of each TXP epitope (Fig. 2B) confirms the selectivity of the antibody with regard to a free C terminus. Despite a certain likelihood that each of the TXP antibodies recognizes epitopes with an amino acid exchange, these results indicate a selective enrichment of target analytes that bear the respective signature peptide. The acceptance of amino acid exchanges in epitope recognition might be attributed to the polyclonal character of the antibodies. In summary, the consistency of the epitope mapping results suggests that generated TXP antibodies are excellently suited for immunoaffinity enrichments.

**TXP Affinity Enrichment MS with HEK293 Cell Lysates**—Next, the enrichment capability of the anti-AMTR and anti-LGYR TXP antibodies was tested in an immunoaffinity enrichment MALDI MS work flow. Tryptic digests of differentially treated HEK293 cells were generated, and the complex peptide mixtures were analyzed by immunoaffinity MS. HEK293 cells were either treated or not treated with SB216763, which prevents the phosphorylation of β-catenin by GSK3 (27) and hence the degradation of β-catenin (28). Therefore, the cellular amount of β-catenin was expected to be higher in SB216763-treated cells. Protein extracts from both cell cultures were enzymatically digested using trypsin and were applied to anti-AMTR and anti-LGYR antibodies immobilized on solid affinity column supports. Captured peptides were eluted and subsequently analyzed using MALDI MS. The individual peptides were identified with MALDI-TOF/TOF. After TXP affinity enrichment, MALDI-TOF/TOF MS was used to detect and verify both LGYR and AMTR β-catenin signature peptides (Table I, A and B). A Mascot ion score of 43 was obtained for the AMTR peptide, and a score of 232 was obtained for the LGYR peptide. The direct MS analysis of trypsin-digested HEK293 lysates from stimulated and unstimulated cells without affinity enrichment did not lead to the identification of any of the identified peptides including the two β-catenin-specific signature peptides.

As an example, Fig. 3 displays the MALDI MS spectra of tryptically digested GSK3 inhibitor-treated cells without (A) and with (B) affinity enrichment using the anti-LGYR antibodies. The β-catenin signature peptide TEPMAWNETADLGL-DIGAQPGLGYR (m/z 2804.3) could be detected and fragmented in the enriched material from the treated cells only (Table IB). Thus, it was possible to independently detect β-catenin signature peptides by using two different TXP antibodies in an immunoaffinity MS work flow.

Besides the β-catenin fragments, several other trypic peptides were identified in the enriched samples using the anti-AMTR antibodies (Table IA). As expected from the epitope characterization experiments, two peptides contained the targeted C terminus AMTR, and 18 peptides contained the AXTR motif at the C terminus. At the second position, serine, threonine, valine, alanine, glutamine, histidine, asparagine, tyrosine, or glycine was found instead of methionine in the C-terminal sequence of the captured peptides. The presence of all these amino acids apart from asparagine had been predicted by epitope mapping using the positional scanning peptide libraries. In summary, in the MS spectra of the untreated sample, it was possible to assign 11 signals of 47 MS signals (S/N > 10) within a mass range of 800 and 5000 Da (Table IA). In the spectra of the treated sample, 20 signals of 66 MS signals (S/N > 10) could be assigned by MALDI MS/MS.

The use of anti-LGYR antibodies enabled the MS/MS-based identification of 12 peptides of 51 MS signals in the untreated cell culture lysate (Table IB). Of 55 MS signals, 16 peptides could be assigned in the sample derived from the treated cell culture (Table IB). Nine peptides had amino acids other than tyrosine, and one had isoleucine instead of leucine in their C-terminal sequence. Thus, eight of 18 captured peptides contained the targeted C terminus LGYR. However, the amino acids phenylalanine, leucine, alanine, and glycine were identified instead of tyrosine in some peptides. The replacement of tyrosine by amino acids other than phenylalanine and leucine/isoleucine was not expected from the epitope characterization experiments using the positional scanning peptide libraries (Fig. 2B).

In addition, the TXP antibodies had a strong preference for arginine at the C terminus because no other amino acid was found at the C terminus of all peptides analyzed in the biological samples. This is consistent with the results of the epitope characterization.

In terms of reproducibility, all parent ions whose fragments could be identified by MS/MS came up in both technical replicates of the respective sample either treated or untreated. Overall, 21 of the identified 38 peptides appeared in both samples. Only three of these 21 peptides that appeared in the untreated samples captured by the anti-AMTR antibody could not be confirmed by MS in both technical replicates. Most likely this is due to the fact that the ion signals were close to the detection limit of the mass spectrometer. Taking these data together, we could achieve reproducible results despite the polyclonal character of the antibodies used (see Table I, A and B).

In summary, the generated TXP antibodies were able to enrich peptides containing the targeted AMTR or LGYR sequence as well as peptides that harbored one other amino acid at a certain position in the TXP epitope. In total, we were able to identify 38 signature peptides using only two TXP antibodies for immunoaffinity enrichment. Thereby, it could be demonstrated that antibodies having targeted multispecificity can be applied in MS-based multiplexed protein analysis.

**DISCUSSION**

Peptide-centric strategies have become powerful tools for the identification of differentially expressed proteins in whole proteome analyses. However, a major bottleneck in such approaches is the need for excessive separation and sampling steps prior to MS readout. It is an inherent issue in mass spectrometry-based protein analysis that reasonable sensitiv-
| m/z | Protein ID | Peptide sequence | Peptide identified by MS/MS in enriched peptide pools using anti-AMTR (A) and anti-LGYR antibodies (B) | Non-treated | SB216763-treated |
|-----|------------|------------------|-------------------------------------------------------------------------------------------------|-------------|------------------|
| 1268.7 | Host cell factor | AMTR HSHAVSTAAAMTR | Yes | 25 | 11 | 25 | Yes | 25 | 11 | 25 | Yes |
| 4727.4 | Catenin β-1 | AMTR GNPEEDEVDTSOLYVYEEOQFSQSFTEVQGAAQGOYAMTR² | Yes | 43 | 40 | 14 | Yes | 43 | 40 | 14 | Yes |
| 104.86 | Replication factor C subunit 5 | AMTR GIPLSFASTR | RFC5 | Yes | 36 | 28 | 25 | Yes | 43 | 37 | 25 | Yes |
| 159.98 | Uncharacterized protein C10orf12 | AMTR NSSHQANLPATAPSTR | CG012 | Yes | 57 | 47 | 24 | Yes | 57 | 47 | 24 | Yes |
| 163.48 | Kinesin heavy chain | AMTR TOMDQEELLAATR | KINN | Yes | 55 | 41 | 24 | Yes | 55 | 41 | 24 | Yes |
| 176.93 | Mitochondrial import receptor subunit TOM40 homolog | AMTR ASDOLOHGVEFASTR | TOM40 | Yes | 84 | 72 | 23 | Yes | 84 | 72 | 23 | Yes |
| 360.83 | Nuclear inhibitor of protein phosphatase 1 | AMTR LEPHKQQPQIDSTVSGASTR | PP1R98 | Yes | 147 | 127 | 20 | Yes | 147 | 127 | 20 | Yes |
| 894.5 | Vacuolar protein sorting-associated protein 18 | AMTR SFVIATTR | VPS18 | Yes | 32 | 15 | 25 | Yes | 32 | 15 | 25 | Yes |
| 1390.7 | WD repeat protein 85 | AMTR NGTWLQATAATTR | WDR85 | Yes | 108 | 85 | 24 | Yes | 108 | 85 | 24 | Yes |
| 2336.3 | Polyhomeotic-like protein 3 | AMTR SPSDPHVSVPPPPLLPAATR | PHC3 | Yes | 46 | 37 | 21 | Yes | 46 | 37 | 21 | Yes |
| 840.5 | Pyruvate kinase isozymes M1/M2 | AMTR APIIAVTR | KPYM | Yes | 41 | 12 | 19 | Yes | 41 | 12 | 19 | Yes |
| 1183.6 | Protein-disulfide isomerase A3 precursor | AMTR LAPEYEAAATR | PDIA3 | Yes | 34 | 19 | 26 | Yes | 34 | 19 | 26 | Yes |
| 1191.6 | E3 ubiquitin-protein ligase HUWE1 | AMTR NLCYHAQTR | HUWE1 | ND | Yes | 39 | 29 | 23 | Yes | 39 | 29 | 23 | Yes |
| 1162.6 | 28 S ribosomal protein S2, mitochondrial | AMTR DCGEYAHTR | RT02 | ND | Yes | 64 | 62 | 25 | Yes | 64 | 62 | 25 | Yes |
| 1108.5 | Heme oxygenase 1 | AMTR DCEQVPQQPTVYVQALFDFDPQEDGELGFR | HMOX1 | ND | Yes | 35 | 32 | 23 | Yes | 35 | 32 | 23 | Yes |
| 1382.9 | Transmembrane protein 41B | AMTR SQGLAHHTVPSQDGAAATR | TM41B | Yes | 46 | 27 | 24 | Yes | 46 | 27 | 24 | Yes |
| 805.6 | GTP-binding protein RhoB | AMTR IAILGYR | RHEB | Yes | 25 | 17 | 26 | Yes | 25 | 17 | 26 | Yes |
| 908.5 | Nucleoporin p58;p45 | AMTR EQYLYGR | NUPL1 | Yes | 27 | 10 | 26 | Yes | 27 | 10 | 26 | Yes |
| 951.8 | α-Adducin | AMTR TLQNLGYR | AODG | Yes | 25 | 11 | 24 | Yes | 25 | 11 | 24 | Yes |
| 1026.6 | Protein transport protein Sec33A | AMTR YLQELGGR | SEC33A | Yes | 47 | 20 | 24 | Yes | 47 | 20 | 24 | Yes |
| 1077.6 | ATP-dependent RNA helicase DDX42 | AMTR LNNGGGGLGYR | DDX42 | Yes | 40 | 20 | 25 | Yes | 40 | 20 | 25 | Yes |
| 1183.6 | THO complex subunit 6 homolog | AMTR VDIDTNLGYR | THOC6 | Yes | 33 | 13 | 25 | Yes | 33 | 13 | 25 | Yes |
| 1552.8 | Zinc finger MIZ domain-containing protein 1 | AMTR VAAQQQDFDDLGFR | ZMIZ1 | Yes | 46 | 27 | 24 | Yes | 46 | 27 | 24 | Yes |
| 2804.3 | Catenin β-1 | AMTR TEPMAWNETADGLDGLGAQGEPYLGR² | Catenin β-1 | Yes | 232 | 220 | 18 | Yes | 232 | 220 | 18 | Yes |
| 1599.9 | Catenin β-1 | AMTR QPELPEVIAMLGFR (methionine oxidized) | Catenin β-1 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 2633.2 | Ubiquitin fusion degradation protein 1 homolog | AMTR QVEQHESTEDEVSDGIGAYAEGLFR² | UFDB1 | Yes | 84 | 77 | 21 | Yes | 84 | 77 | 21 | Yes |
| 3381.6 | Growth factor receptor-bound protein 2 | AMTR DIEQVPOOQPTVYQALFDFDPQEDGELGFR | GRB2 | Yes | 34 | 14 | 18 | Yes | 34 | 14 | 18 | Yes |
| 932.6 | Programmed cell death protein 2-like | AMTR FVDQDLGR | CTBL1 | Yes | 34 | 21 | 19 | Yes | 34 | 21 | 19 | Yes |
| 129.8 | Programmed cell death protein 2-like | AMTR AAVLKVPLLGLR + acetyl (N terminus) | PDD2L | Yes | 27 | 18 | 20 | Yes | 27 | 18 | 20 | Yes |
ity can only be achieved by reducing sample complexity. The introduction of an immunoaffinity step as part of knowledge-independent or knowledge-based shotgun approaches improves speed and sensitivity of MS-based peptide identification and quantification. Enriched peptides can be further analyzed in single reaction monitoring/multiple reaction monitoring mode (9) or directly by MALDI MS (29). Consequently, the SISCAPA (9) or immunoaffinity MALDI (29) strategy allows the number of processed samples to be increased, which is a prerequisite for biomarker validation approaches. However, they rely on the availability of appropriate capture reagents, namely peptide-specific antibodies. The generation of pep-

| C terminus                        | Peptide sequence          | Protein ID                                      | Mascot score | Distance to next hit | Identity threshold | Parent ions detected in replicates |
|-----------------------------------|---------------------------|------------------------------------------------|--------------|---------------------|-------------------|-----------------------------------|
| Non-treated                      |                           |                                                |              |                     |                   |                                   |
| 1742.0                            | LGAR FGTVLTEHVAAAELGAR    | Deoxyribonucleoside 5'-monophosphate N-glycosidase | 30           | 27                  | 21                | Yes                              |
| 1781.9                            | LGAR LAEMPADSGYPAYLGAR    | V-type proton ATPase catalytic subunit A         | 75           | 60                  | 24                | Yes                              |
| 1409.8                            | LGGR VLATTFDPYLGGR        | Heat shock 70-kDa protein 4                     | 24           | 10                  | 23                | Yes                              |
| 2000.0                            | IGYR FLYGENMPPQDAEIGYR    | Dual specificity protein kinase                  | 41           | 29                  | 23                | Yes                              |
| 1698.8                            | LGLR AWDQEAEGAGPELGLR     | Aldehyde dehydrogenase family                   |              |                     |                   |                                   |
| 16 member A1                      |                           |                                                |              |                     |                   |                                   |

**Table I—continued**

Fig. 3. Shown are MALDI MS spectra of tryptically digested GSK3 inhibitor-treated cells without (A) and with affinity enrichment using the anti-LGYR antibodies (B). The β-catenin signature peptide TEPMAWNETADLGIDLIGAQGELPYLGYR (m/z 2804.374) can be detected in the enriched material only. The fragment ions derived from m/z 2804.374 are shown in spectrum C. Additionally, seven other peptides harboring LGYR in the C terminus were identified by MS/MS. These peptides were only detectable in MALDI MS spectra of the immunoaffinity-enriched samples. AU, arbitrary units.
tide-specific antibodies in the dimension of a proteome as recently suggested requires huge financial resources and is a challenging project (12).

The work presented here shows that it is not only possible to use one antibody for detecting one single analyte, but also a “one antibody–multiple analyte” concept is feasible. In this proof-of-concept study, we demonstrated that groups of peptides sharing the same short epitope can be isolated by immunoaffinity enrichment and analyzed by MS/MS. Of interest is the fact that these enriched peptides share a common motif that contains valuable additional sequence information to support MS/MS-based peptide identification based on peptide probability scores. The knowledge of immunoaffinity capture specificity and fragment masses from MS/MS experiments represents two statistically independent data sets that should help to increase the validity of peptide identification in shotgun approaches in the future.

To get detailed information on the applicability and availability of such multispecific antibodies, polyclonal antisera against two different C-terminal peptides, LGYR and AMTR, were raised. These terminal sequences are found in tryptic fragments derived from β-catenin, which was chosen as the model target protein in this study. Antibody preparations obtained after affinity purification were thoroughly characterized using positional scanning peptide libraries for epitope specificity before being used for immunoaffinity enrichment. A workflow to enrich peptides from whole proteome digests using TXP antibodies was established and applied for the analysis of digests of cell line lysates. The use of the TXP antibodies enabled us to directly detect β-catenin and other signature peptides without further chromatographic separation.

As the first step in this study, the in-depth characterization of the two TXP antibodies with respect to their epitope specificity was performed. Affinity-purified polyclonal antibodies against the C-terminal AMTR and LGYR epitopes were analyzed using two independent approaches: antibody binding and peptide capture assays. The use of positional scanning peptide libraries allowed precise determination of the epitope and the effect of a single amino acid exchange in the targeted epitope. Thereby, the definition of the recognized epitope (20) (Fig. 2A) was possible. The thorough investigation of the recognized epitope for the AMTR antibodies revealed an AXTR motif in which the methionine has a minor effect on peptide binding. In the case of the anti-LGYR antibodies, amino acid variabilities emerged for Leu, Gly, and Tyr. Nevertheless, the target sequence LGYR revealed the highest binding signal. These results were corroborated using a peptide capture assay, and the role of distinct amino acids relevant for epitope recognition could be determined as complementary information (Fig. 2B).

With detailed knowledge on epitope recognition, the generated TXP antibodies were used to analyze tryptically digested HEK293 cell lysates. Both antibodies, targeting the AMTR or LGYR motif, were capable of enriching their respective β-catenin target peptide. As expected, additional peptides containing AMTR or LGYR termini were found; one peptide containing terminal AMTR and seven tryptic fragments harboring the LGYR epitope could be confirmed by MS/MS (Table I, A and B). Additional peptides were enriched, and the MS/MS spectra were used to define their sequence. For the antibody generated against AMTR, peptides with an AXTR motif were enriched, and for the anti-LGYR antibody, additional peptides with amino acid exchanges at position 3 (tyrosine) were found. At the moment it is not clear whether the observed cross-reactivity is associated with the polyclonality of the antibodies, and monoclonal antibodies are being generated to clarify this issue. Using monoclonal antibody technology, the hybridoma supernatants may allow screening for antibodies displaying a higher epitope specificity, and clones with a clearly defined recognition pattern can be selected for expansion. However, the current experiments showed that the generated polyclonal TXP antibodies are capable of enriching peptide subgroups in an immunoaffinity MS work flow.

To get a better idea of the value of our results, a thorough inspection of major peptide databases was performed. UniProt (version 57.14), Global Proteome Machine Database (GPMDB; February 2010) (30) and PeptideAtlas (built March 2009) (31, 32) were used to predict peptides containing a C-terminal LGYR or AMTR sequence in tryptically digested human samples (see Table II; details are described in supplemental Table 3, A and B). Of note, the GPMDB and PeptideAtlas are empirical databases that contain peptides that have been detected in MS-based experiments. The in silico digest (complete tryptic digest with no mis- and missed cleavages) of UniProt revealed 28 peptides containing AMTR and 71 peptides with LGYR at the C terminus. GPMDB and PeptideAtlas searches yielded much lower numbers. For the LGYR C terminus, nine peptides were found in the PeptideAtlas, and 18 were found in the GPMDB. Peptides with an AMTR C terminus

| Table II | Overlap of sequences identified in enriched peptide pools with calculated (UniProt) and actually observed peptides (PeptideAtlas and Global Proteome Machine (GPM)) |
|---------|----------------------------------------------------------------------------------------------------------|
| Number of AMTR peptides | Number of LGYR peptides |
| Calculated (UniProt)a | 28 | 71b |
| Evidence on transcript level (UniProt)a | 10 | 19 |
| Evidence on protein level (UniProt)a | 18 | 51 |
| Global Proteome Machine (downloaded February 2010) | 4 | 18 |
| PeptideAtlas (built March 2009) | 2 | 9 |
| Identified in TXP fractions | 2 | 8 |
| Overlap with GPM | 2 | 4 |
| Overlap with PeptideAtlas | 1 | 3 |
| In TXP fractions only | 0 | 3 |

a Version 57.14/Swiss-Prot/Homo sapiens.
b One protein uncertain.
Targeting Peptide Termini

occur four times in the GPMDB and only twice in the PeptideAtlas. Our analysis of HEK293 did show that two peptides with an AMTR C terminus and eight peptides with an LGYR C-terminal sequence were identified. The comparison of the results of the immunoaffinity enrichment using the two antibodies shows four of 18 LGYR-containing peptides and two of four AMTR peptides with overlap to GPMDB. For the PeptideAtlas, the degree of overlap was higher. One of two AMTR peptides was found, and three of nine LGYR peptides are deposited in this database. Interestingly, three LGYR peptides were identified that have not been annotated in these empirical databases.

The overlap with the empirical databases might not seem spectacular, but it has to be taken into account that peptides identified by the TXP approach were from one cell line only using a MALDI-based MS readout. The deposited data combine results from various sample sources, from different laboratories, and from electrospray- and MALDI-based MS readouts. Because of the complementary nature of electrospray ionization and MALDI, further experiments using electrospray-based mass spectrometry might result in a higher degree of overlap. At the moment, reasons for the observed differences in sequence overlap cannot be clearly assigned to technical issues, to biological specimen, or to limited capacity of the prepared affinity columns. Differences may also be associated with imperfect trypsin digestion, an issue all peptide-centric protein analysis approaches are facing.

Our approach of using immunoaffinity enrichment of classes of peptides that share short epitopes opens interesting possibilities when thinking about proteome-wide expression analysis. Bioinformatics calculations (33) revealed that an optimized set of only 100 antibodies should be able to recognize peptides derived from 10,000 different proteins (based on the UniProt database). Thus, the TXP concept could represent an efficient strategy to enrich peptides prior to MS-based analysis, and a high ratio of analytes per antibody could make it useful for immunoaffinity-coupled MS-based analysis targeting a large number of analytes. In a focused analysis in which small sets of proteins are to be detected, a TXP antibody-based enrichment could show advantages over conventional strategies as well. A bioinformatics analysis showed that six TXP antibodies may be sufficient for the detection of the 12 members of the human catenin family (34) (including α-catenin). In this example, the C-terminal sequence AVSK occurs in four proteotypic peptides derived from the four different catenin family members, δ-catenin, δ-catenin 2, plakophilin-4, and armadillo repeat gene deleted in velocardiofacial syndrome. Thus, this one antibody could be used to enrich different peptides derived from a small set of related proteins. Here, the concept is obviously less efficient compared with targeting a larger group of analytes. Interestingly, our bioinformatics analysis showed that this immunoaffinity approach could also be useful for the analysis of larger protein families that consist of homologue proteins. It should be possible to obtain tryptic peptides that share a short terminal epitope, which in this case is derived from a conserved region of the protein families. Differences in the remaining part of the peptides are present and will allow the identification of the different protein family members. For this application, the concept of group-specific enrichment seems to be especially useful, and the value of this type of analysis for protein families is under investigation.

The work performed in this study stays well within this scope, and the aim here was to show that it is possible to detect one protein reliably by using different antibodies generated against short TXP epitopes. We demonstrated the principal feasibility of the TXP concept by raising polyclonal rabbit antibodies against the C-terminal peptide sequences AMTR and LGYR and using them as multispecific capture reagents in a rapid MALDI MS-based immunoaffinity approach. Antibody characterization revealed a defined epitope specificity, good reproducibility of peptide capture, and minor cross-reactivities despite their polyclonal character. The two TXP antibodies were used in a simple protein analysis workflow and allowed the parallel detection of 38 peptides derived from 37 proteins. For a closer understanding of the performance and the limitations of our work flow, several issues have to be addressed in the future. Issues like analyte recovery, capture efficiency, and sensitivity still have to be investigated using stable isotope-labeled standards for each of the target peptides and compared with either targeted approaches like SISCAPA or non-targeted multidimensional LC-MS/MS setups. Moreover, it has to be investigated whether the various target peptides sharing the same epitope compete for the antibody binding, thus affecting detection limit of these peptides. We carefully evaluated the influence of the distinct amino acid residues in the epitope of the antibodies, but we cannot exclude that epitope-flanking amino acids and peptide length exert an effect on the immunoprecipitation efficiency. Assuming a similar affinity of the antibody to the various peptides might allow parallel IP and quantification by using stable isotope-labeled standard target peptides, e.g. in combination with multiple reaction monitoring. These experiments will allow the determination of the dynamic range of peptide concentrations that can be measured in the captured peptide pool of one TXP antibody. Based on such data, it will be possible to predict how far peptides derived from lower abundance proteins can be quantified and detected in the presence of high abundance proteins. Of course, monoclonal antibodies having a defined affinity would be of advantage for these experiments. Our data indicate that the generation of TXP antibodies for immunoaffinity-based proteome studies could be feasible, enabling knowledge-based as well as knowledge-independent immunoaffinity-MS/MS protein analyses in the future without the need for generating individual antibodies for each and every analyte.
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This article contains supplemental Table 3.

§ Both authors contributed equally to this work.

** To whom correspondence should be addressed. Tel.: 49-7121-51530-820; Fax: 49-7121-51530-816; E-mail: poetz@nmi.de.

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