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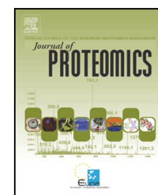

Evaluation of inflammation-related signaling events covering phosphorylation and nuclear translocation of proteins based on mass spectrometry data

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ABSTRACT

Peripheral blood mononuclear cells are important players in immune regulation relying on a complex network of signaling pathways. In this study, we evaluated the power of label-free quantitative shotgun proteomics regarding the comprehensive characterization of signaling pathways in such primary cells by studying regulation of protein abundance, post-translational modifications and nuclear translocation events. The effects of inflammatory stimulation and the treatment of stimulated cells with dexamethasone were investigated. Therefore, a previously published dataset accessible via ProteomeXchange consisting of 6901 identified protein groups was reevaluated. These data enabled us to comprehensively map the c-JUN, ERK5 and NF-κB signaling cascade in a semi-quantitative fashion. Without the application of any enrichment, 3775 highly confident phosphopeptides derived from 1249 proteins including 66 kinases were identified. Efficient subcellular fractionation and subsequent comparative analysis identified previously unrecognized inflammation-associated nuclear translocation events of proteins such as histone-modifying proteins, zinc finger proteins as well as transcription factors. Profound effects of inflammatory stimulation and dexamethasone treatment on histone H3 and ZFP161 localization represent novel findings and were verified by immunofluorescence. In conclusion, we demonstrate that multiple regulatory events resulting from the activity of signaling pathways can be determined out of untargeted shotgun proteomics data.

Significance: Relevant functional events such as phosphorylation and nuclear translocation of proteins were extracted from high-resolution mass spectrometry data and provided additional biological information contained in shotgun proteomics data.

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1. Introduction

Signaling pathways are based on complex networks of interacting proteins displaying different functionalities, involving ligands, receptors, transcription factors and effector molecules. The dysregulation or activation of proteins or protein complexes within such pathways may be sufficient to induce pathophysiological processes like inflammation. On this account, protein members of signaling pathways may represent targets for therapeutic interventions. Although the use of immunoblots and gene expression analysis methods are still the most common ways to systematically evaluate signaling pathways, high-end proteomic techniques are getting more and more attractive for this field of research [1–3]. Mass spectrometry (MS)-based proteomics is the method of choice with regard to complex protein mixtures covering a large dynamic concentration range [4]. The analysis of various sample types such as tissues and cells at different functional states was accomplished by large-scale proteome profiling strategies, thereby contributing to the draft map of the human proteome [5]. Actually, modern proteomics aims at the comprehensive assessment of cell and tissue states covering not only protein identification and quantitation but also the determination of post-translational modifications [6].

However, the main approaches for the analysis of phosphoproteins are based on the selective enrichment of phosphopeptides by

Abbreviations: Dex, dexamethasone; FDR, false discovery rate; IL, interleukin; JUN, transcription factor AP-1; LFQ, label-free quantification; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; NFKB1, NF-kappa-B p105 subunit; PAK, serine/threonine-protein kinase PAK; PBMCs, peripheral blood mononuclear cells; PEP, posterior error probability; PHA, phytoheamagglutinin; PKA, cAMP-dependent protein kinase catalytic subunit alpha; PRIDE, proteomics identification database; RT, room temperature; RUNX2, runt-related transcription factor 2; STAT3, signal transducer and activator of transcription 3; TiO2, titanium dioxide; ZFP161, zinc finger protein 161 homolog.

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immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO_2) or antibody-based enrichment strategies [7–9]. The challenge of shotgun proteomics becomes evident considering the phosphopeptide identification rate within such experiments. Only if the dynamic range of measurement is improved, the sequence coverage will increase and low abundant phosphopeptides successfully identified [10]. With such improvements, in depth analysis of relevant physiological as well as pathophysiological mechanisms may become feasible with shotgun approaches.

Inflammation is such a physiological process involved in almost all kinds of human pathologies and of enormous molecular complexity. Different cell types act in a concerted fashion; different kinds of molecules such as proteins and lipids are key mediators and may represent important biomarkers as well as drug targets. A comprehensive understanding of the molecular processes will be the basis to improve clinical management especially of chronic inflammation states. However, such a comprehension is not easy to achieve as all kinds of molecular signaling mechanisms are involved in inflammation such as ligand receptor binding, phosphorylation events, activation of transcription factors, modulation of enzymatic activities and many more. We have investigated inflammation processes in human white blood cells by proteome profiling, thus focusing on up- and down-regulation of proteins including cytokines, transcription factors, proteases and redox regulators [11].

The aim of this study was the critical assessment of current capabilities of shotgun proteomics regarding the analysis of inflammation-related signaling events such as inflammation-associated phosphorylation and nuclear translocation of proteins. Therefore we have re-evaluated the shotgun analysis dataset freely accessible via ProteomeXchange [12] comprising proteome profiles of secreted, cytoplasmic and nuclear fractions from control, inflammatory activated as well as dexamethasone-treated inflammatory activated PBMCs [11]. The application of novel analysis strategies indeed reproduced known molecular events and also provided new insights regarding inflammation-associated phosphorylation and translocation events of proteins in PBMCs.

2. Material and methods

A dataset published previously by us [11], available via ProteomeXchange with identifiers PXD001415-23 was used for this study. Briefly, this dataset was obtained from three samples each of control, inflammatory activated as well as dexamethasone-treated inflammatory activated PBMCs, of which secreted, cytoplasmic and nuclear proteins were extracted, respectively, which were further processed separately. Normalization to the total protein content was achieved by digesting 20 μg of protein from each respective sample with trypsin. The resulting peptides of each sub-cellular fraction were subjected to a subsequent nanoLC-MS/MS analysis. To this end, peptides were injected into a Dionex Ultimate 3000 nano LC-system coupled to a QExactive orbitrap mass spectrometer equipped with a nanospray ion source (Thermo Fisher Scientific, Austria). Peptides were first loaded on a 2 cm × 75 μm C18 Pepmap100 pre-column (Thermo Fisher Scientific, Austria) at a flow rate of 10 μl/min using mobile phase A (98% H2O, 2% ACN, 0.1% FA). Elution from the pre-column to a 50 cm × 75 μm Pepmap100 analytical column (Thermo Fisher Scientific, Austria) and subsequent separation was achieved at a flow rate of 300 nl/min using a gradient of 8% to 40% mobile phase B (80% ACN, 20% H2O, 0.1% FA) over 235 min for the analysis of the cytoplasmic and nuclear fractions, and over 95 min for secretomes. MS scans were performed in the range from m/z 400–1400 at a resolution of 70,000 (at m/z = 200). For MS/MS scans, a top 12 method for cytoplasmic and nuclear fractions and a top 8 method for secretomes were achieved through HCD fragmentation at 30% normalized collision energy and a resolution of 17,500 (at m/z = 200). All samples were measured in technical duplicates.

2.1. MaxQuant data analysis for the evaluation of subcellular fractionation and protein translocation

For protein identification as well as label-free quantitative (LFQ) data analysis, the open source software MaxQuant 1.3.0.5 including the Andromeda search engine and the Perseus statistical analysis package [13,14] was used. Search criteria included the use of the SwissProt database (version 012013 with 20,264 entries) and a peptide tolerance of 25 ppm as well as a maximum of 2 missed cleavages. Furthermore, carbamidomethylation on cysteins was set as fixed modification and methionine oxidation as well as N-terminal protein acetylation as variable modifications. For positive protein identification, a minimum of two peptide identifications per protein, at least one of them unique, were required. Match between runs was performed using a 5 min match time window and a 15 min alignment time window. Common contaminants were included in the search for appropriate FDR determination. All peptides and proteins were meeting an FDR < 0.01. Assessment of peptide abundances using MaxQuant was achieved by the integration of MS1 peaks in a three-dimensional fashion based on a distinct m/z, retention time window and intensity. Relative peptide abundances were summed up to obtain LFQ values as quantitative measure for protein abundance, which is normalized by applying a global optimization procedure in order to achieve the least overall proteome variation [15]. Statistical analysis was performed using Perseus (version 1.3.0.4), filtering proteins for reversed sequences, common contaminants and a minimum of three independent experimental identifications per protein. To determine significant differences of protein abundance between cytoplasmic and nuclear fractions, missing values were replaced by random numbers obtained from normal distributions at the detection limit and LFQ values were submitted to a two-sided *t*-test with a significance threshold of $p < 0.05$, which was applied for control, inflammatory activated as well as dexamethasone-treated inflammatory activated PBMCs. For multi-parameter (MP) correction, a permutation based FDR calculation was performed. Proteins passing an FDR < 0.05 are indicated (Supplementary Tables S3).

For the evaluation of subcellular fractionation, these fold-change values of the control PBMCs obtained from the *t*-test statistics between cytoplasmic and nuclear fraction were sorted from the highest to the lowest and categorized into 7 groups using Excel. Proteins of each group were also classified as “nucleus” and/or “cytoplasm” according to Uniprot keyword attributes and visualized using pie charts. Furthermore, LFQ-values of three biological replicates including two technical measurements were used for heat map analysis using R [16].

For the determination of protein translocation, these proteins were sorted from the highest to the lowest difference values between nucleus and cytoplasm from control cells and plotted as black line. The corresponding difference values derived from the inflammatory activated cells were plotted in dark-red, whereas the respective difference values derived from dexamethasone-treated inflammatory activated cells were plotted in light-red (Fig. 2a). Data evaluation was performed using Excel.

2.2. Immunofluorescence

PBMCs were purified with written consent and approval of the Ethics Committee of the Medical University of Vienna (Application 2011/296 by C.G. entitled “Charakterisierung von entzündlich aktivierten Zellen des peripheren Blutes…”) as described previously [11,17]. Briefly, 30 ml of non-coagulated whole blood were collected in 6 ml CPDA tubes (Greiner Bio-One GmbH, Austria) and immediately processed by diluting it 1:2 with RPMI1640 medium (Gibco, Life Technologies, Austria) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (ATCC, LGC Standards GmbH, Germany). Afterwards, the diluted blood suspension was carefully overlaid on Ficoll Paque (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) and centrifuged at 500 g for 20 min at 24 °C. The interphase was then collected and washed

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