Quantitative phosphoproteome analysis for the detection of early alterations of signal transduction by dioxin (TCDD)

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Overview and Conclusion

- TCDD is an ubiquitinous chemical pollutant and is the most potent carcinogen in animal experiments
- SILAC in combination with SIMAC and TiO_2 is a straightforward method for isolation, identification and quantitation of phosphorylated peptides
- This approach provided new insights of the early actions of TCDD *in vivo* that has not been described before

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an extremely toxic, ubiquitous environmental pollutant and regarded the most potent chemical carcinogen ever tested in experimental animals. Most of its biological effects are mediated by binding of TCDD to the cytosolic Ah receptor (AHR). Following ligand binding, the liganded AHR translocates to the nucleus, where it heterodimerizes with the related protein ARNT. The AHR/ARNT complex then acts as a transcriptional activator of the expression of a battery of AHR-responsive genes. In addition, TCDD has been reported to rapidly cause AHR-dependent alterations in signal transduction that are independent of changes in gene expression but also implicated in the toxicity of dioxin. These early, and partially cell type-specific, effects of TCDD preceding transcriptional activation of target genes are poorly understood. In the present study, we have therefore conducted a quantitative phospho proteomic study on TCDD-induced alterations of global protein phosphorylation in 5L rat hepatoma cells, a well-known model for the investigation of mechanisms of dioxin toxicity.

Results

Identification of the phosphoproteome of 5L cells

- From a starting material of 800 µg , 5648 phosphorylated peptides with 6573 different phosphorylation sites were identified and quantified, using the database IPI rat
- These sites correspond to 2156 different phospho-proteins
- Most of the detected phosphorylation sites have not been described before

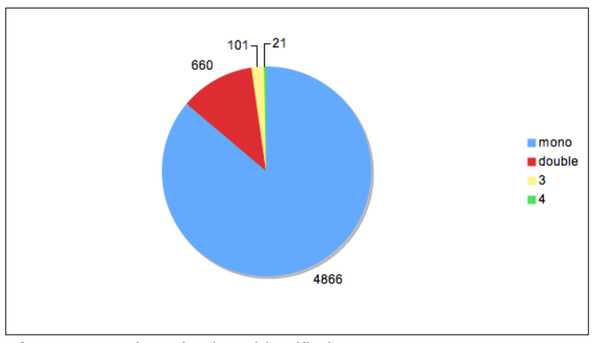

Figure 1: Number of unique identified phosphopeptides. The diagram splitted in mono- and multi phosphorylated peptides

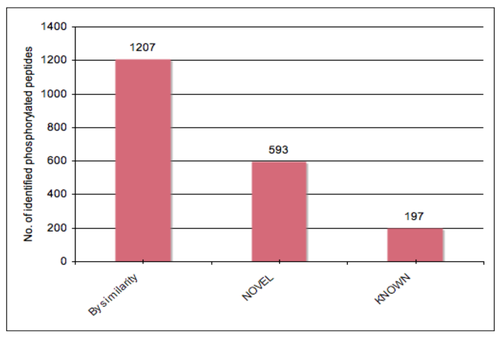

Figure 2: Overview of known, novel or "known by similarity" phospho sites based on UniProtKB. Only 655 phospho-proteins (with 1997 phosphorylated peptides) identified with IPI could be mapped to a UniProt Accession number.

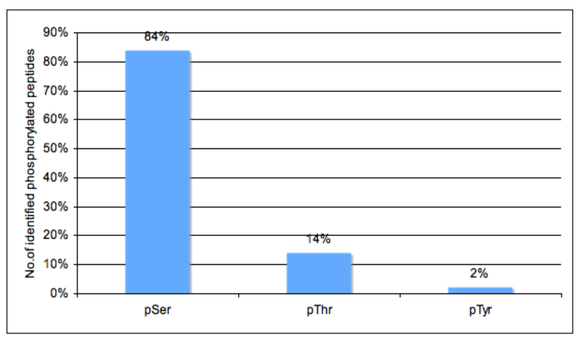

Figure 3: Overview ot the distribution of pSer, pThr and pTyr found for this data set

- Distribution of pSer:pThr:pTyr is 84%:14%:2%
- consistent with other observation in large-scale observations

Quantitative and Bioinformatic analysis

- 22 different significantly overrepresented serine kinase motifs and 12 threonine kinase motifs were identified (using the program motif-x)
- Kinase motifs for CAM2 and CK2 were significantly overrepresented
- 5 Serine and 5 threonine kinase motifs could not be mapped map to known kinase motifs

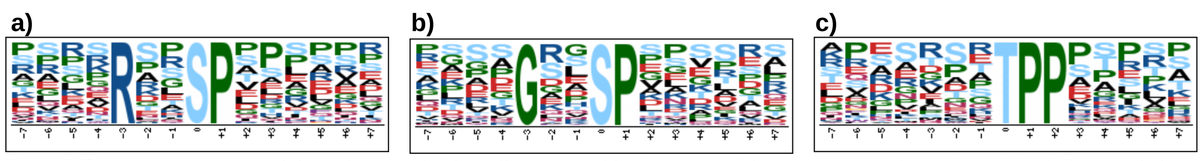

Figure4: Sequence logo representations, the height of the letter represent the frequency of the occurrence of the amino acids; a) Represents the most significant motif for the identified serine phosphorylated peptides. The motif could be fitted to CAM2. b) This analyzed motif could not been fit to a known kinase motif. c) Shown is the most significant motif for threonine phosphorylated peptides (Proline-directed Kinase substrate motif).

In total, 11 proteins with statistically significantly altered protein phosphorylation (q>0.05) are detected. Furthermore 21 proteins with altered phosphorylation (FC > 1.5) identified in only one experiment were detected. The regulated peptides play a role in for example:

- **Transcriptional co-regulators**
- **Calcium signalling**
- **Inflammatory response**

Experimental Design

5L rat hepatoma cells were grown in RPMI medium w/o arginine and lysine. For stable isotope labelling, $^{12}C_6$-lysine plus $^{15}N_4$-arginine and $^{13}C_6$-lysine plus $^{13}C_6{}^{15}N_5$-arginine were added to the medium for DMSO-treated (control) cells and TCDD-treated cells, respectively. This labelling scheme was employed to circumvent quantitation problems associated with the conversion of arginine to proline (Van Hoof et al., *Nature Meth*. 4:678 (2007)). After 5 cell cycles, the cells were serum starved for 24 hours and treated with 1 nM TCDD for 30min, 1h and 2h. After treatment, the cells were lysed and the proteins precipitated using ice cold acetone. After precipitation, the proteins were redissolved in 6M urea/2M thiourea, diluted 5-fold in 50 mM ABC buffer and digested with trypsin for ~16h. The phosphopeptides were sequentially enriched by the SIMAC strategy thereby separating mono- and multi-phosphorylated peptides. Peptides of the multi-phosphorylated fraction were directly identified and quantitated on a LTQ Orbitrap XL. The mono-phosphorylated fraction was prefractionated by hydrophilic interaction chromatography (HILIC) and further enriched for phosphorylated peptides using TiO_2 and analyzed on a LTQ Orbitrap XL. Data were searched against a target-decoy rat database using the Mascot algorithm. For quantitation the MSQuant software was used. All MS and MS/MS spectra were manually validated to exclude peptides with low quality (low S/N ratio, low scans, overlapping peaks). Identification of statistically significantly "regulated" peptides was performed with the R-packages "limma" and "q-Value". All peptides with a q-value below 0.05 were accepted as significantly regulated. Phosphopeptides detected only once per time point were regarded as "regulated" when the regulation factor (RF) exceeded 1.5 ($\geq$1.5 or $\leq$0.67).

Examples of regulated proteins

ARNT (Aryl hydrocarbon receptor nuclear translocator)

- Ser77 phosphorylation found as TCDD-regulated at 1h and 2h (RF 2.44 and 3.67, respectively)
- First observation of this phosphorylation *in vivo;* previously only observed *in vitro*, there catalyzed by casein kinase II
- ARNT/ARNT homodimers bind to a palindromic DNA sequence termed "E-box element", an enhancer not recognized by the AHR/ARNT heterodimer that activates the dioxin-responsive element (DRE, XRE); i*n vitro* phosphorylation prevents binding of the ARNT to the E-box, but not binding of the AHR/ARNT heterodimer to the DRE
- **TCDD-induced increase in Ser77 phosphorylation enhances recruitment of ARNT to DREs and activation of dioxin-responsive genes?**

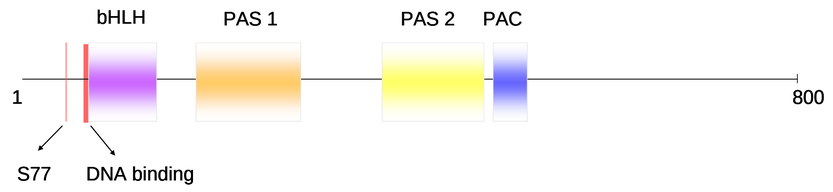

Figure 5: Shematic view of the domain structure of ARNT. ARNT is a basic-Helix-Loop-Helix–Per-ARNT-Sim (bHLH–PAS) protein with an N-terminal bHLH domain, two PAS domains and one PAC domain. The identified phospho site is S77.

Similar to AHNAK nucleoprotein

- Phosphorylation corresponding to Ser213 of the human orthologue found as TCDD-regulated at 1h (RF 1.89 and 2.08) and 2h (RF 1.71 and 1.49)
- AHNAK regulates calcium currents through L-type calcium channels and activates phospholipase Cγ
- **AHNAK phosphorylation and localization through TCDD-induced intracellular calcium changes and so activation of the phospholipase Cγ/PKC signalling?**

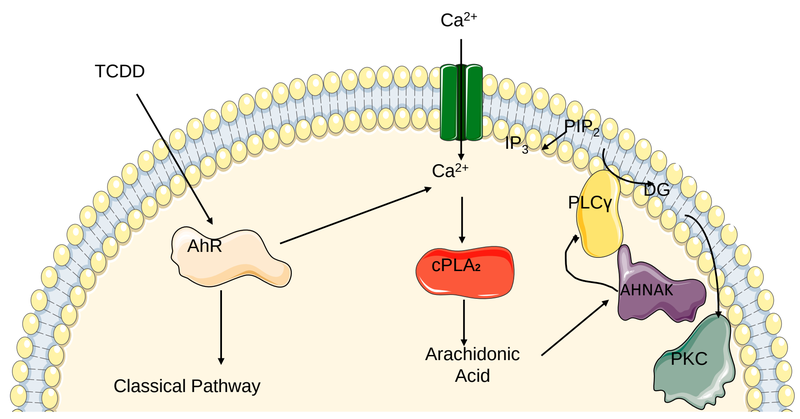

Figure 6: Shematic digram shows the nongenomic TCDD pathway. It is known that due to TCDD a Ca^{2+} influx appeares and so $cPLA_2$ and PKC are activated. AHNAK could now explain the activation of PKCγ..

- Other peptides from AHNAK were identified in LC MS/MS but not as regulated
- Furthermore the same peptide with a different phospho site were identified
- Both phospho sites are phosphorylated by different kinases
- Spectra annotation could clearly determine the localization of both phosphorylation sites (see below)

LPSG**S**GAASPTTGSAVDIR
y15/b5

LPSGSGAA**S**PTTGSAVDIR
y11/b9

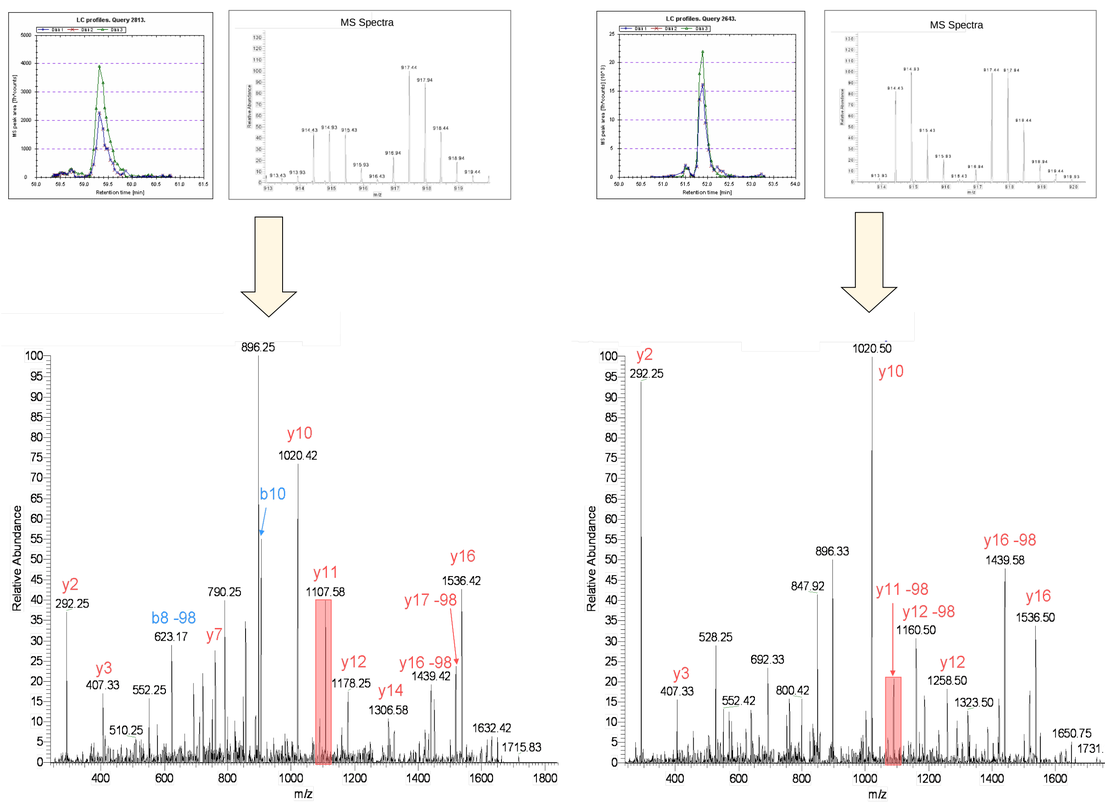

Figure 7: Phospho site specific quantitation. Extracted ion chromatogram and MS-Spectra from the peptides LPSG**pS**GAASPTTGSAVDIR and LPSGSGAA**pS**PTTGSAVDIR as well MS/MS spectra annotation for the two different identified phospho peptides.

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