Methods for the analysis of phosphorylation sites in the DivIVa protein

Mass spectrometric analysis of phosphorylation sites

Protein samples were digested with TPCK Immobilized Magnetic Trypsin (Clontec, Saint-Germain-en-Laye, France) for 60 or 90 min. The digestions were stopped by removing the magnetic beads and addition of TFA (Rathburn Chemicals Ltd, Walkerburn, Scotland) to a final concentration of 0.5%. For LC-MS/MS analysis, a sample aliquot was applied via a nanoAcquity UPLC®-system (Waters, Manchester, UK) running at a flow rate of 250 nL min^{-1} to an LTQ-ORBITRAP™ mass spectrometer (Thermo Fisher, Waltham, MA, USA). Peptides were trapped using a pre-column (Symmetry® C18, 5µm, 180 µm x 20 mm, Waters) which was then switched in-line to an analytical column (BEH C18,1.7 µm, 75 µm x 250 mm, Waters) for separation. Peptides were eluted with a gradient of 3-40% acetonitrile in water/0.1% formic acid at a rate of 0.67% min^{-1}. The column was connected to a 10 µm SilicaTip™ nanospray emitter (New Objective, Woburn, MA, USA) attached to a nanospray interface (Proxeon(Thermo), Odense, Denmark) for infusion into the mass spectrometer. The mass spectrometer was operated in positive ion mode at a capillary temperature of 200 °C. The source voltage and focusing voltages were tuned for the transmission of MRFA peptide (m/z 524) (Sigma-Aldrich, St. Louis, MO). Data dependent analysis was carried out in orbitrap-IT parallel mode using CID fragmentation on the 5 most abundant ions in each cycle. For detection and analysis of phosphopeptides, multistage activation was used with neutral loss m/z of 48.99 and 32.66 (for 2+ and 3+ charged ions).

The orbitrap was run with a resolution of 30,000 over the MS range from m/z 350 to m/z 1800 and an MS target of 10^6 and 1 s maximum scan time. Collision energy was 35, and an isolation width of 2 was used. Only monoisotopic 2+ and 3+ charged precursors were selected for MS2. The MS2 was triggered by a minimal signal of 1000 with an AGC target of $3x10^4$ ions and 150 ms scan time using the chromatography function for peak apex detection. Dynamic exclusion was set to 1 count and 60 s exclusion time with an exclusion mass window of ±20 ppm. MS scans were saved in profile mode while MSMS scans were saved in centroid mode.

For targeted analysis of specific potential phosphopeptides, their masses (m/z) were included in an inclusion list for triggering MS2 fragmentation, and no dynamic exclusion and no peak apex detection was used.

Raw files were processed with MaxQuant version 1.3.0.5 (Cox and Mann, 2008; http://maxquant.org) to generate re-calibrated peaklist-files which were used for a database search using an in-house Mascot® 2.4 Server (Matrix Science Limited, London, UK). Mascot-mgf files were generated from MaxQuant apl-files using suitable perl scripts.

Mascot searches were performed on the *Streptomyces coelicolor* protein sequences downloaded from Uniprot (www.uniprot.org, 8093 sequences, 04042013) to which the mutated DivIVa sequence had been added, or for simplifying searches on a small database containing the target sequences (wild-type and mutated DivIVa) in a background of 100 random *E. coli* sequences using 5 ppm precursor tolerance, 0.5 Da fragment tolerance, and oxidation (M) as well as phosphorylation (STY) as variable modifications.

Mascot search results were imported into Scaffold 3.6.3 including an Xtandem! search for evaluation and further into ScaffoldPTM™ (version 2.0, proteomsoftware.com, Portland, OR, USA) for analysis of phosphorylation sites using the Ascore algorithm implemented in ScaffoldPTM. The spectra shown for visual inspection were annotated in the ScaffoldPTM programme.