

Systematic PTM analysis of a set of recombinant protein kinases using LC-MS/MS data

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ThP 34 Slot 660

Overview

A set of more than 30 recombinant protein kinases has been characterized on the basis of LC-MS/MS data. Kinases are proteins of interest as they are known to regulate the majority of cellular pathways.

The data was interpreted using the PTM-Explorer software which allows automatic screening for a set of modifications (including phosphorylation), amino acid substitutions, unsuspected large measurement errors, enzyme non-specificity and unknown mass shifts.

Numerous modifications were found, such as methylation, pyroglutamate formation and methionine oxidation. Internal significance analysis revealed various different phosphorylation sites in the kinase samples. Phosphorylation of the kinases in Sf9 cells as well as autophosphorylation were successfully monitored.

Introduction

Protein kinases contain a characteristic domain, make up one of the largest "superfamilies" of eukaryotic proteins. Due to their versatility, they play many key roles in biology and disease.

Phosphorylation by protein kinases is recognized as a major mechanism by which many activities of eukaryotic cells are regulated, including proliferation, gene expression, signal transduction, metabolism, motility, membrane transport, and apoptosis. Thus dysregulated kinase activity is a frequent cause of disease, particularly cancer, and therefore kinases are a major target for research activities.

Because of the profound effects of protein kinases on cellular processes, their activity is highly regulated. Kinases are regulated themselves by phosphorylation as well as via autophosphorylation, by binding of activator or inhibitor proteins, or by controlling their cellular location. There is therefore a major interest in phosphorylation sites and phosphorylation status of kinases themselves which is the main focus of the present analysis.

The classification of protein kinases is mainly based on their substrate specificity. There are three major groups: serine/threonine kinases, receptor tyrosine kinases and cytoplasmatic tyrosine kinases. [1, 2]

References

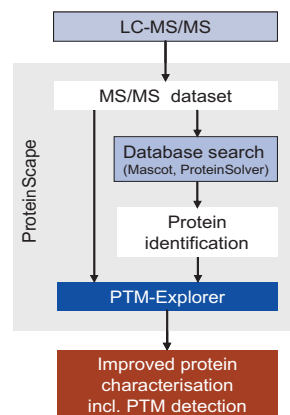
1. Hanks SK. Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. *Genome Biol.* 2003;4(5): 111.
2. Hanks SK, Hunter T. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* 1995; 9: 576-596.

We analysed a set of protein kinases, produced by ProQinase GmbH, for post translational modifications. The interest is directed towards the phosphorylation status because of its relevance for the function. After tryptic digestion and nanoLC-MS/MS the generated MS/MS-spectra were analysed by a novel algorithm named PTM-Explorer. This interpretation tool enables the screening for any kind of modification, e.g. phosphorylation, in a given data set.

Methods

Sample preparation and MS analysis

The analysed protein kinases were recombinantly produced by ProQinase GmbH in Sf9 cells. After tryptic digestion of the purified protein samples MS/MS spectra were acquired by nanoLC-MS/MS (1 hour gradient from 10% to 90% Acetonitrile) using the Switchos/Ultimate System (Dionex) and the LCQ Deca XP (Thermo). The datasets were imported into the proteome bioinformatics platform ProteinScape V1.3 (Bruker Daltonik GmbH, Bremen, Germany; Protagen AG, Dortmund, Germany)(see also poster of Körting et al. TP 34 Slot 643). A sequence database (NCBI nr) search for protein identification was performed using the Mascot (www.matrixscience.com) and ProteinSolver (Bruker Daltonik GmbH, Bremen, Germany; Protagen AG, Dortmund, Germany) search engines.



PTM-Explorer analysis

PTM-Explorer searches are performed on target protein sequences. Here, the kinase sequences that were identified in previous sequence database searches were investigated in the PTM-Explorer analysis (Figure 1).

Using PTM-Explorer spectra were screened for a set of modifications (Figure 2), amino acid substitutions, enzyme non-specificity and unknown mass shifts. In all searches methionine oxidation was regarded as additional variable modification. The unknown mass shift mode aimed at detection of mass shifts that are not covered by the set of searched modifications. It can detect unsuspected large measurement errors, too. Input to the PTM-Explorer algorithm is provided by filling in a web form, target sequences and spectra are automatically submitted to the algorithm (see also poster of Chamrad et al. MP 15 Slot 335 for more information on PTM-Explorer).

Figure 1. Overview on the MS/MS data interpretation. The spectra were imported into ProteinScape, the proteome bioinformatics platform, at first. From there protein identification via database searches including efficient result evaluation can be performed in an highly automated manner. PTM-Explorer is a part of ProteinScape and is used for detailed characterization and modification detection of proteins from MS/MS data. PTM-Explorer searches are usually restricted to the proteins, which have been previously identified.

Modification	Mass shift	Modification	Mass shift
Acetylation (N-term)	42.0106	Methylation (N-term)	14.0157
Acetylation (K)	42.0106	Myristoylation (K)	210.1984
Amidation (C)	-0.9840	Myristoylation (N-term)	210.1984
Beta-methylthiolation (D)	45.9877	N-acyl diglyceride cysteine (C)	788.7258
Biotinylation (K)	226.0776	N-pyruvic acid 2-iminyl (CVK)	70.0468
Carbamylation (K)	43.0058	O-GlcNAc (NST)	203.0794
Citrullination (R)	0.9840	Palmitoylation (CKST)	238.2297
Deamidation (NQ)	0.9840	Phosphorylation (STYHCD)	79.9799
Di-methylation (KR)	28.0532	Pyridoxal phosphate (K)	229.0140
Farnesylation (C)	204.1878	Pyro-glutamine (Q)	-17.0305
Formylation (C)	27.9949	Phosphopantetheine (S)	339.0780
Geranyl-geranyl (C)	272.2504	Sulfation (Y)	79.9568
Gamma-carboxylation (DE)	43.9898	Sulphone (M)	31.9988
Glucosylation (KNTWCYR)	162.0528	Tri-methylation (KR)	42.0797
Glucosylation (N-term)	162.0528		
Glutathione disulfide (C)	305.3076	Chymotryptic cleavage	-
Hydroxylation (DKNP)	15.9949	No enzyme (one end)	-
Mannosylation (C)	162.0528	Unknown shift	variable
Methylation (CDEHKNGRST)	14.0157	Substitution	variable

Table 1. Using PTM-Explorer the MS/MS spectra of the kinase samples have been searched for the above modifications. The modifications were tested sequentially and methionine oxidation was always regarded as variable experimental modification. Besides non-specific cleavage, unknown mass shifts and possible amino acid substitutions were tested also.

Results

Overview on the detected modifications

Nearly 38 % of the peptides that were identified by PTM-Explorer are not covered by standard sequence database searches (i.e. in this case a search that regards only variable methionine oxidation). The main identifications came from experimentally introduced Na⁺ adducts and phosphorylations. There is also a large group of spectra that obviously contain a sequence tag of the analysed kinase, but the suggested sequence does not match the parent mass (unknown mass shift). A large amount of spectra were cleaved non-tryptic on one side.

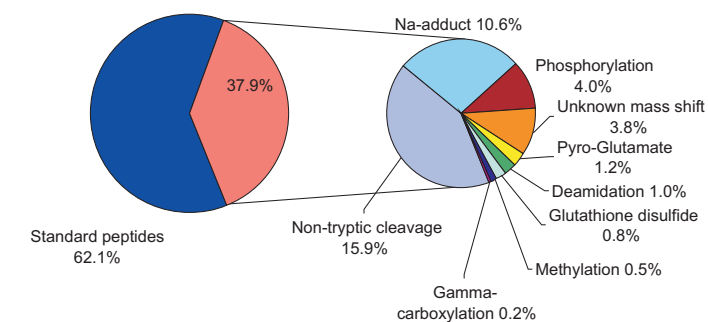


Figure 2. Classification of the peptides, which have been detected by PTM-Explorer. The group of standard peptides are tryptic peptides, which may also contain methionine oxidation. Only peptides, which obtained a significance value >30 are shown. All modified peptides have been validated manually.

Characterisation of kinase proteins

39 different phosphorylation sites were detected by PTM-Explorer in 40 kinase samples (Table 2). The corresponding spectra have been revealed by internal significance analysis of PTM-Explorer and were additionally validated manually. As phosphorylated peptides mostly show an unfavourable fragmentation behaviour leading to weak spectra, the existence of the phosphorylation is not always absolutely clear and should be verified by additional experiments. In some cases the exact position can not be deduced from the MS/MS spectrum. Most of the protein modifications were covered by more than one spectrum.

Kinase protein	Accession	PTM	Pos	Described in Swissprot
Lyn	g14505055	Phosphorylation	Y-306	
		Phosphorylation	Y-316	
		Phosphorylation	Y-473	
MST 4	g115011880	Gamma-carboxylation	E-127	
		Phospho-phanthethine	S-279	
		Phosphorylation	S-416	
MAPK 11	g120988526	Phosphorylation	T-180	activates the kinase, probable
		Phosphorylation	Y-182	activates the kinase, probable
		Phosphorylation	T-241	
		Phosphorylation	S-243	
		Phosphorylation	S-(252,253)	
PIM	g168165002	Phosphorylation	S-99	
		Phosphorylation	S-(352,353)	
		Phosphorylation	Y-315	
BLK	g133469882	Phosphorylation	Y-315	
EPHA3	g1125387	Methylation	S-912	
ERBB2	g154792098	Acetylation	S-1144	
Aurora-C	g151471708	Acetylation	K-(158,159)	
		Phosphorylation	S-191	
MAPK 14	g14503069	Phosphorylation	T-123	
		Phosphorylation	T-180	activates the kinase, probable
		Phosphorylation	Y-182	activates the kinase, probable
		Phosphorylation	Y-258	
SAK	g123243309	Phosphorylation	T-263	
		Phosphorylation	S-818	
FRK	g155958775	Phosphorylation	Y-46	
		Phosphorylation	Y-497	
HCK	g156205101	Phosphorylation	Y-187	
		Phosphorylation	Y-309	
		Phosphorylation	Y-470	
PAK7	g156205345	Phosphorylation	S-82	
		Phosphorylation	S-(233,226)	
		Phosphorylation	S-278	
		Phosphorylation	S-288	
		Phosphorylation	S-(342,345)	
PDK	g115082370	Phosphorylation	S-(802,803)	
		Phosphorylation	(S,T)(35-37)	by similarity, autocatalysis
B-Raf	g175516780	Phosphorylation	S-242	
EPHA2	g160810087	Phosphorylation	S-408	
		Glutathione disulfide	C-748	
EPHA1	g14823217	Phosphorylation	S-897	
TSK2	g147678705	Phosphorylation	S-621	
		Hydroxylation	K-10	
EPHA4	g14758280	Methylation	637-639	
EPHB1	g14104413	Glutathione disulfide	C- 692	
		Hydroxylation	K-624	
NEK6	g147605963	Glutathione disulfide	C-314	
		Phosphorylation	S-120	
VEGF-R2	g155666094	Phosphorylation	T-144	
		Glutathione disulfide	C-308	
		Phosphorylation	S-(472,474)	S-472, by similarity

Table 2. Summary of the detected post-translational modifications. Only very few can be currently found in the literature. In some cases the exact position could not be deduced from the MS/MS spectrum e.g. Phosphorylation at PDK: (S,T)(35-37) means that the phosphorylation can be at S or T between the positions 35 and 37.

The following spectra are both examples for detected phosphorylated peptides. In both cases the unmodified peptide version is also present.

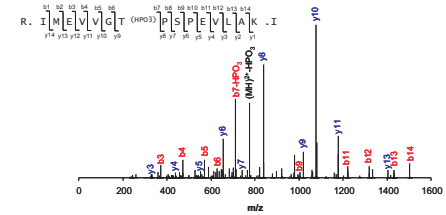


Figure 3. MS/MS spectrum showing the phosphorylation at T-241 in MAPK 11.

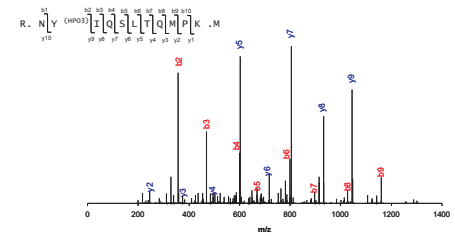


Figure 4. MS/MS spectrum showing the phosphorylation at Y-258 in MAPK 14.

Conclusion

Kinase autophosphorylation was successfully monitored, using PTM-Explorer software for MS/MS based characterization of recombinant kinase proteins. Altogether 39 different phosphorylation sites were discovered in 40 analysed kinase proteins.

Besides phosphorylation other modifications such as methylation or acetylation were measured. About 40% of the detected peptides were not standard peptides (tryptic peptides including possible methionine oxidation). Most of these stem from one side unspecific cleavage or are Na⁺ adducts.

Results indicate that additional knowledge can be gained from the large amount of MS/MS spectra that remain unidentified after standard sequence database searches, if second pass search tools such as PTM-Explorer are used.

Application of PTM-Explorer is advantageous in the environment of quality control in biotechnology processes or therapeutic proteins or peptides. The grade of automation and flexibility suits well to the routine analytics of recombinant proteins. Careful validation can be limited to less than 30min for an LC-MS/MS analysis.