MASS SPECTROMETRIC CHARACTERIZATION OF PROTEINS INVOLVED IN OXIDATIVE STRESS RESPONSE IN YEAST

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INTRODUCTION

Intracellular concentration of reactive oxygen species such as hydroperoxides is narrowly set by specialized pathways to preserve cellular integrity. In *Saccharomyces cerevisiae*, the transcription factor Yap1 is involved in peroxide oxidative stress response. In normal conditions, Yap1 is located in the cytoplasm because it is rapidly exported out of the nucleus by the nuclear receptor Crm1/Xpo1. When cells are exposed to hydroperoxides, Yap1 cannot interact with the receptor anymore and accumulates inside the nucleus. It then elevates the expression of genes encoding most antioxidants (figure 1).

We aimed to study the molecular mechanisms associated with Yap1 using mass spectrometry.

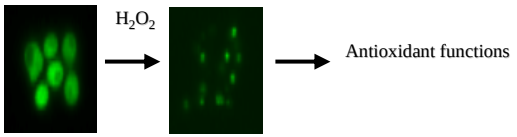


Figure 1: Nuclear relocalization of GFP-tagged Yap1 in response to H_2O_2

Material and methods

Protein samples

Proteins extracted from the *S. cerevisiae* YPH98 strain [2] (*MATa, ura3-52, lys2-801^{amber}, ade2-101^{ochre} trp1-Δ1 leu2-Δ1*) and from mutant strains were purified in the presence of N-EthylMaleimide NEM, so that cysteines in the SH form were modified. The recombinant protein Myc-Yap1 corresponding to Yap1 carrying 9 Myc epitopes at its N-terminal end was purified on a Sepharose 4B resin covalently linked to the monoclonal anti-Myc 9E10 antibody. Purified species were then separated by SDS-PAGE according to previously described protocols.[1,3]

LC-MS/MS and MS/MS analyses

Half of the proteins in gel slices were digested by trypsin, while the other half was reduced by dithiothreitol (DTT) and alkylated with iodoacetamide before digestion as described.[4]

For the identification of Gpx3, the tryptic digest of the Yap1^{C303A} high MW band (not reduced and alkylated by iodoacetamide before digestion) was analyzed by nanoscale capillary liquid chromatography – tandem mass spectrometry (LC-MS/MS), using a Famos-Switchos-UltiMate capillary LC system (LC Packings, Amsterdam) connected to an ESI-QqTOF hybrid mass spectrometer (Q-TOF2, Micromass, Manchester, UK). Chromatographic separations were conducted on a reversed-phase (RP) capillary column (Pepmap C18, 75 µm i.d., 15 cm length, LC Packings) at a 200 nL/min flow, with a linear gradient from 100% A (H_2O/acetonitrile/formic acid, 96/4/0.1, v:v) to 50% B (H_2O/acetonitrile/formic acid, 10/90/0.085, v:v) in 50 min, followed by a 15-min flush at 100% B. LC-MS/MS data was obtained in automatic mode and converted into a .PKL file using Masslynx software (Micromass), and submitted to the search software Mascot (http://www.matrixscience.com/). Proteins were identified by comparison of experimental data to the NCBInr database.

For the identification of disulfide bridges, tryptic peptides from Yap1 and Gpx3 were manually analyzed by tandem mass spectrometry (MS/MS) on the Q-TOF2. Ions with masses corresponding to expected disulfide-linked peptides or to their reduced and alkylated counterparts were detected, selected and fragmented.

Memento

Yap1 is a 72.5 kDa protein which contains 6 cysteines at positions 303, 310, 315, 598, 620 and 629.

Gpx3 is a 20 kDa protein which contains 3 cysteines at positions 36, 64 and 82.

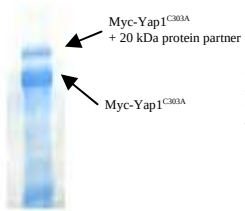
Yap1^{C303A} refers to the mutant of Yap1 in which cysteine 303 was replaced with an alanine

References

(1) Delaunay, A., Pflieger, D., Barrault, M.-B., Vinh, J. and Toledano, M. B. (2002). Cell 2002 111: 471-481. (2) Sikorski, R. S., et Hieter, P. (1989). Genetics 122, 19-27. (3) Delaunay, A., Isnard A.-D. and Toledano, M.B. (2000) EMBO J. 19, 5157-66. (4) Shevchenko, A., Wilm, M., Vorm, O., et Mann, M. (1996). Anal. Chem. 68, 850-858.

RESULTS

Yap1 interacts covalently with a 20kDa protein



Immunoprecipitation of the protein Myc-Yap1 was performed on the protein extract of hydrogen peroxide-treated yeast cells and enabled the co-purification of a second protein species which migrated at a higher MW than Myc-Yap1. This species was immunoreactive towards the anti-Myc antibody, which proved that it corresponded to the association of Yap1 with a partner of about 20 kDa. This gel band was then called 'Yap1 high MW band'. This protein association was induced by hydrogen peroxide (H2O2) treatment. Only a weak percentage of the Yap1 pool was involved in this linkage (Figure 2); the study of the native and the different mutant strains (punctual mutations of each cysteinyl residue of *Yap1*) showed that this protein was most present in the mutant strain *Yap1C303A*.

Figure 2: Migration of Myc-Yap1C303A on a non-reducing gel

Identification of Yap1 partner by LC-MS/MS analysis

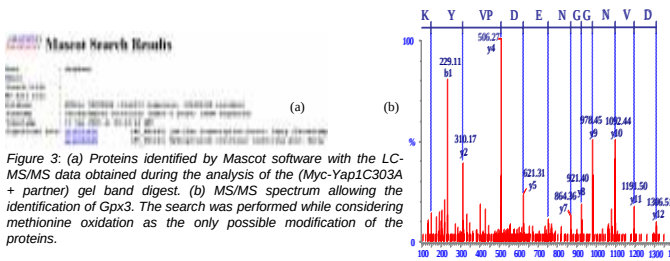
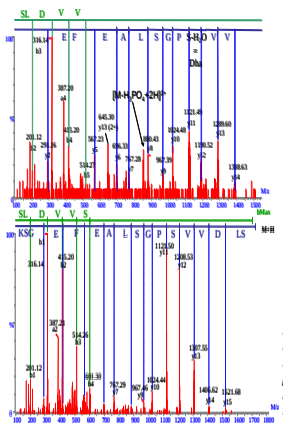


Figure 3: (a) Proteins identified by Mascot software with the LC-MS/MS data obtained during the analysis of the (Myc-Yap1C303A + partner) gel band digest. (b) MS/MS spectrum allowing the identification of Gpx3. The search was performed while considering methionine oxidation as the only possible modification of the proteins.

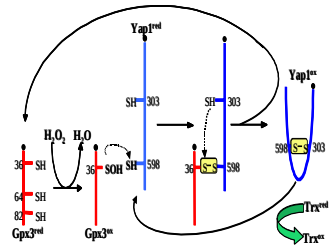
The Yap1 high MW band purified from *Yap1C303A* mutant cells was used for this LC-MS/MS analysis.
Yap1 : 40% protein sequence coverage (16 different peptides were fragmented)
Gpx3 : 27% protein sequence coverage (peptides 26-35, 96-108 and 142-160 were identified).

Spectra misinterpreted by Mascot software



Possible cause of misinterpretation	Further search	Results
Yap1 phosphorylation state has been observed to change in response to H_2O_2 [3]	Phosphorylation considered as a possible modification	Peptide 9-25 from Yap1^{C303A} phosphorylated on residue Ser14
The protein has been purified in the presence of NEM	NEM introduced as a personalized modification of cysteines to be considered in the search	Peptide 611-626 from Yap1 in which Cys620 is alkylated with NEM
The recorded protein sequences may be different from the actual sequences (mutagenesis)	Manual interpretation of high-quality spectra	- peptides corresponding to the Myc epitopes, - peptide 279-305 from Yap1^{C303A} containing the mutation Cys303Ala

Figure 4: Fragmentation spectra corresponding to the phosphorylated (A) and unphosphorylated (B) peptide 9-25 from Yap1 acquired during automated LC-MS/MS analysis of the tryptic digest of Myc-Yap1^{C303A}-Gpx3. The phosphorylation on residue Ser14 is revealed on the MS/MS spectrum: the ion $[M\text{-}H_3PO_4\text{+}2H]^{2+}$ is detected and the loss of the 14th residue during fragmentation appears as a loss of dehydroalanine (dhA, 69.02 Da).



Suggested model of the response mechanism to a hydroperoxide stress.

Figure 5: Proposed model mechanism describing the oxidation of Yap1

MS/MS analysis revealed Yap1-Gpx3 intermolecular bridge

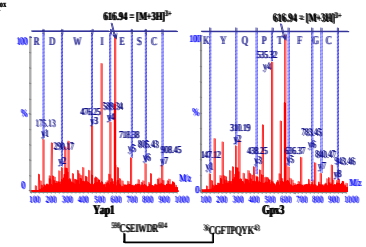


Figure 6: Fragmentation of a triply-charged species corresponding to the tryptic peptides 598-604 from Yap1 and 36-43 from Gpx3 covalently linked by a disulfide bridge (M = 1847.8 Da; m/z = 616.94). The sequences of both peptides can be fully read on the spectrum.

MS/MS analysis reinforced the hypothesis of an intramolecular disulfide bridge between Yap1 cysteines 303 and 598

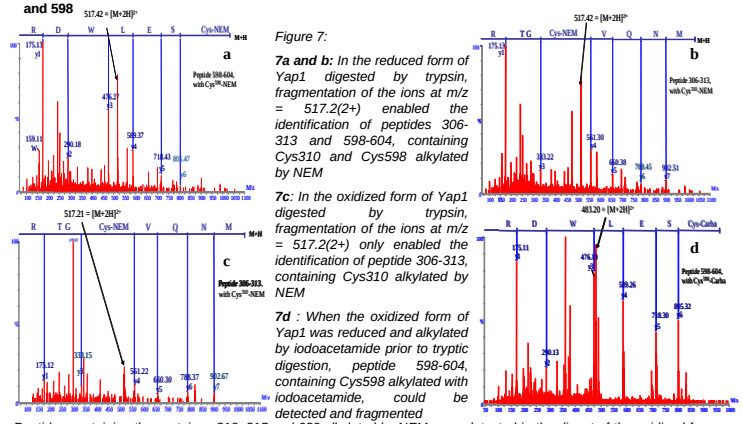


Figure 7:

***7a and b:** In the reduced form of Yap1 digested by trypsin, fragmentation of the ions at m/z = 517.2(2+) enabled the identification of peptides 306-313 and 598-604, containing Cys310 and Cys598 alkylated by NEM*

***7c**: In the oxidized form of Yap1 digested by trypsin, fragmentation of the ions at m/z = 517.2(2+) only enabled the identification of peptide 306-313, containing Cys310 alkylated by NEM*

***7d** : When the oxidized form of Yap1 was reduced and alkylated by iodoacetamide prior to tryptic digestion, peptide 598-604, containing Cys598 alkylated with iodoacetamide, could be detected and fragmented*

Peptides containing the cysteines 310, 315 and 620 alkylated by NEM were detected in the digest of the oxidized form of Yap1. These three cysteines thus do not seem to be involved in an intramolecular disulfide bridge. On the contrary, Cys598 is likely to take part in such a bridge according to the results of figure 7. Cysteines 629 and 303 could not be detected.

Conclusion – Perspectives

1) A 20 kDa protein partner of Yap1 was identified as the glutathione peroxidase Gpx3. **2)** A phosphorylation site on Yap1-Gpx3 was localized at Ser14 of Yap1. **3)** Yap1 and Gpx3 are covalently linked by a disulfide bridge between Cys598 of Yap1 and Cys36 of Gpx3. **4)** There may exist an intramolecular disulfide bridge between Cys303 and Cys598 of Yap1, which can be confirmed by further analysis using other digestion enzymes. **5)** The results of these mass spectrometric experiments prompted a redefinition of the molecular mechanisms triggered in yeast by an oxidative stress.

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