

Analysis of Mitotic Phosphorylation Sites in the Nuclear Pore Complex using a MALDI LTQ Orbitrap Mass Spectrometer

Justin Blethrow¹, Rosa Viner¹, Vlad Zabrouskov¹, and Joseph Glavy²

1: Thermo Fisher Scientific, San Jose, California, USA 2: Stevens Institute of Technology, Hoboken, New Jersey, USA

Overview

Purpose: Identification of mitotic phosphorylation sites in Nuclear Pore (NPC) sub-complexes.

Methods: NPC sub-complexes were immunoprecipitated, and analyzed by MALDI LTQ Orbitrap MS.

Results: Seventeen mitotic phosphorylation sites were identified in NPC sub-complexes and associated proteins.

Introduction

The nuclear pore complex (NPC) is an assembly of ~30 proteins that forms the principal passageway for nucleo-cytoplasmic macromolecular traffic. In higher eukaryotes, the NPC undergoes regulated mitotic disassembly into sub-complexes, a process thought to be enacted largely via phosphorylation. In this study, we used antibodies to isolate mitotic NPC sub-complexes, and mapped mitotic phosphorylation sites in their constituent proteins. An antibody against Nup107 was used to isolate the Nup107-160 sub-complex, and monoclonal antibody mAb414 was used to isolate sub-complexes containing FG-repeat proteins involved in substrate transport through the pore. These complexes were digested and analyzed by high mass accuracy MALDI MS and MS/MS to identify their constituents, and to identify mitotic phosphorylation sites following phosphopeptide enrichment. 17 mitotic phosphorylation sites were identified. Nine of these occurred at sites previously annotated to be phosphorylated in large-scale screens, while eight appear to be wholly novel.

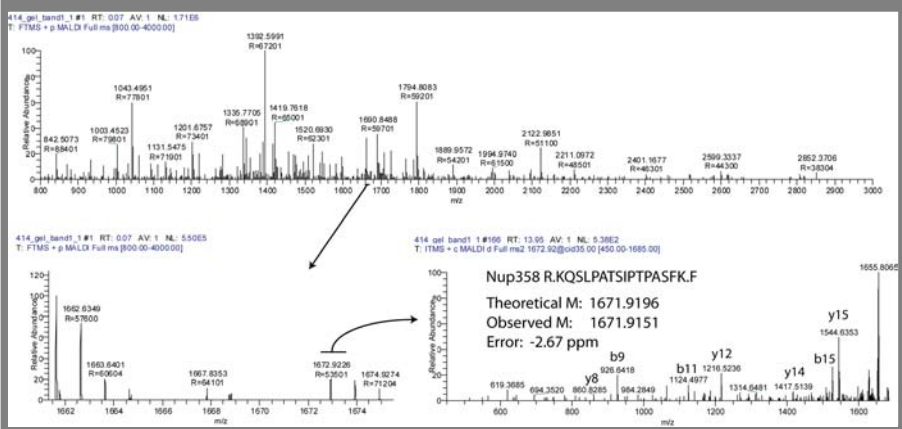
Methods

Cell culture and immunoprecipitations: HeLa cell cultures were synchronized at S-phase entry using a double-thymidine blockade. After release, the cultures were monitored for entry into mitosis and harvested by centrifugation. The frozen pellet was thawed in buffer containing 2% Triton X-100 and protease and phosphatase inhibitors, and lysed by vigorous resuspension. For IPs, the lysate was incubated with 200 mg of protein A-Sepharose per 1 ml of extract for 45 min at 4°C to minimize nonspecific binding. The cleared supernatants were incubated for 2 h at 4°C with 5 mg of mAb414 antibodies (affinity purified against Nup62), bound to protein A-Sepharose. Isolated beads were washed extensively with PBS. The supernatant was incubated for 2 h at 4°C with 5 mg of affinity-purified anti-Nup107 antibodies bound to protein A-Sepharose, then the beads were isolated. Bound proteins were eluted by incubation with SDS-PAGE loading buffer, and stored at -80°C.

Protein digestion and phosphopeptide enrichment: One quarter of the IP samples were loaded separated by SDS-PAGE. The gels were stained with zinc (Bio-Rad) and the top portion of the gel containing proteins above ~60kD was cut into eight MW range sections. These were destained, diced, and digested following standard protocols. Peptides were recovered by incubation with 3 µl of Poros R3 resin in 5% formic acid with rotation for three hours, then removed and eluted with 70% acetonitrile. 5% of the recovered material was spotted on a 384-well MALDI target plate and allowed to dry, then covered with DHB matrix for subsequent analysis. The remaining recovered peptides from each band were incubated with mixing for one hour with ~3 µl of Titansphere titania beads (GL Biosciences) in the presence of 2% TFA and 150 mg/ml DHB in 70% acetonitrile. The beads were packed in a gel-loading tip using Empore C18 (3M) as a frit, washed with the same buffer solution, and the peptides were eluted with 10 ml of ammonium hydroxide / 50% acetonitrile. The eluate was immediately added to 100 ml of 5% aqueous TFA, and passed through a Poros R3 microcolumn made from a gel-loading tip using Empore C18 as a frit. Peptides were eluted with 2 µl of 70% acetonitrile, 5% formic acid onto a 384-well MALDI plate and allowed to dry, then covered with DHB matrix. In parallel, one quarter of each IP sample was precipitated from sample buffer with cold acetone, resuspended, and digested directly.

Mass analysis: The MALDI spots were analyzed using a Thermo Scientific MALDI LTQ Orbitrap™ mass spectrometer. In general, MS analysis was conducted in the FT cell using a resolution of 100K and fragmentation analysis was conducted in the LTQ™ ion trap. For automated data acquisition, automatic gain control (AGC), automatic spectral filtering (ASF) and the crystal positioning system (CPS) were used to automatically locate optimal sample regions and fill the LTQ or C traps appropriately. Software detection of the neutral loss of phosphate was used to automatically perform MS3 analysis of phosphoserine and threonine-containing peptides. The resulting data were analyzed using Mascot, and verified by manual inspection. Phosphopeptides failing to produce IDs in the initial search were subjected to further manual MS³ or MS⁴ analysis (for diphosphopeptides); data were averaged until rich spectra were obtained or no further improvement was possible.

FIGURE 1. MALDI LTQ Orbitrap analysis of peptides recovered from a high weight mAb414 IP gel slice. The upper spectrum shows a full MS scan at 100K nominal resolution; 'R' indicates the observed peak resolution. At bottom left is an inset into the m/z range 1662-1675. The minor peak at m/z 1672.92 was automatically selected for fragmentation in the LTQ; the resulting spectrum, identified as a peptide from Nup358, is shown at the right. Calculated and observed neutral masses are indicated.



Results

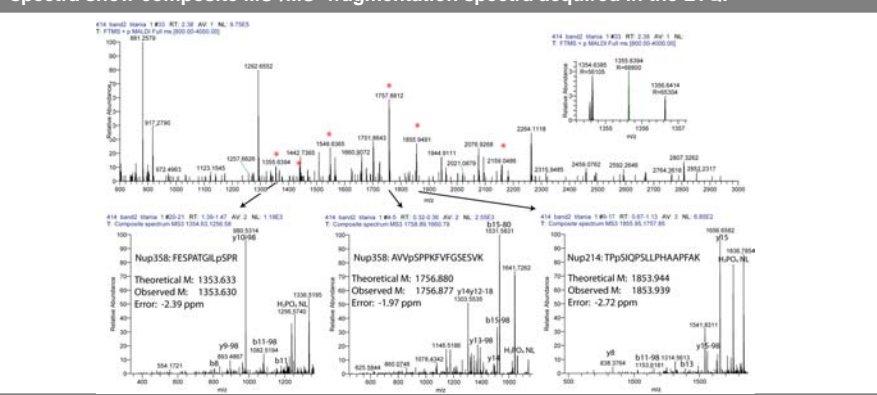
Protein composition of immunoprecipitated NPC sub-complexes: Immunoprecipitation with anti-Nup107 or mAb414 antibodies is expected to provide purification of NPC sub-complexes. Analysis of the peptides recovered from the digested gel slices (Figures 1 and 2) confirmed the successful purification of these targets. In the anti-Nup107 IP, all known components of the Nup107-160 sub-complex over 60kD in weight were identified; gel bands containing proteins below this weight were not processed due to contamination with excess antibody (Figure 2). Intriguingly, several additional proteins were identified, specifically Myosin 9, Myosin 10, and the Clathrin Heavy Chain (CHC). As the Sec13 protein component of the Nup107-160 complex is also a component of clathrin-coated vesicles, the slight presence of CHC may indicated some bridging of these complexes by Sec13. In contrast, Myosin 9 was identified as the most dominant protein in the sample. While some evidence has suggested the presence of a Myosin protein in the intact nuclear pore in *Drosophila* (Ref. 1), and several Myosin-like proteins associate with the pore in yeast (Ref. 2), Myosin is not considered a component of the Nup107-160 sub-complex in vertebrates. Our evidence suggests tight association of Myosin 9 with this sub-complex during mitosis. Alternatively, the anti-Nup107 antibody may simply bind this protein.

FIGURE 2. Proteins identified in the anti-Nup107 and mAb414 immunoprecipitations. Spectra obtained from all processed gel slices were searched in aggregate.

anti-Nup107	Protein	MASCOT Score	% Coverage	mAb414	Protein	MASCOT Score	% Coverage
	Myosin 9	449	25		Nup358	823	26
	Nup133	338	36		Nup214	334	14
	Nup96	183	25		Importin B1	216	24
	Nup107	88	33		Nup88	208	39
	Nup75	57	13		Nup153	166	18
	Myosin 10	42	3		Ran-GAP1	97	30
	Nup160	32	9		Importin A2	93	6
	Clathrin HC	25	2		Nup133	68	19
	Nup88	17	2		Nup54	62	20
					Nup58/45	62	10
					Nup62	58	14
					Hsp7c	39	13
					Grp78	31	9
					SUMO1	20	18

In the case of the mAb414 IP, we identified all of the components of the two major expected sub-complexes, specifically those composed of Nup62/58/54 and Nup358/214/88 (Figure 2). In addition, we detected Ran-GAP1, which is known to associate with Nup358, and also SUMO1. Ran-GAP1 is also known to be modified by SUMOylation, which explains detection of this very small protein when proteins under 60 kD would not be expected. Nuclear import proteins Importin A2 and B1 were also detected. These proteins form a dimeric complex and are known to bind to FG domains during the nuclear translocation process. Two chaperone proteins were identified as well, Hsp7c and GRP78. As FG domains form natively disordered structures, these proteins may serve to stabilize these domains after NPC disassembly. Curiously, the Nup107-160 sub-complex component Nup133 was detected in this IP, and Nup96 was also tentatively identified. This seems to suggest that some fraction of Nup133 associated with the FG-domain complexes rather than Nup107-160 following NPC disassembly.

FIGURE 3. MALDI LTQ Orbitrap analysis of a phosphopeptide-enriched set of peptides derived from digestion of a mAb414 IP gel slice. A full MS scan (m/z range 800-4000 acquired, m/z 800-3000 shown) is shown with phosphopeptides indicated with asterisks. The inset shows resolution of an isobaric contaminant next to the phosphopeptide at m/z 1354.63. The lower spectra show composite MS²/MS³ fragmentation spectra acquired in the LTQ.



Mitotic phosphorylation sites in the NPC sub-complexes: Analysis of the phosphopeptide-enriched fractions (Figures 3, 4) yielded identification of 17 phosphorylation sites in eight proteins. Nine of these sites are annotated as phosphorylated in one or more public databases (ExPASy, HPRD, Phosida) without regard to the cell cycle timing of their phosphorylation. The remaining sites appear to be wholly novel. Comparison of the anti-Nup107 data with that produced in a previous study of mitotic phosphorylation sites in the Nup107-160 sub-complex by one of us (J. Glavy, Ref. 3) revealed that we identified a disparate set of phosphorylation sites, though several of the sites identified here are physically proximal to sites identified in the previous study. As we identified the significant majority of observed presumptive phosphopeptides (those giving a facile neutral loss of 98 daltons), this suggests that we failed to enrich some fraction of mitotic phosphorylation sites in the Nup107-160 sub-complex, or that perhaps there can be some variability in typical site occupancy. On a technical note, it was found that titania-based enrichment of phosphopeptides from digestion of the total IPs was significantly complicated by the presence of antibody. This is due to the fact that immunoglobulin heavy chains are decorated with glycan structures terminated by sialic acid, and titania provides efficient purification of sialic acid-containing species (Ref. 4). Thus, numerous antibody-derived glycopeptides were co-purified along with the phosphopeptides.

FIGURE 4. Summary of phosphopeptides identified in the anti-Nup107 and mAb414 IPs. The observed ions and their corresponding proteins, peptide sequences, and phosphorylation sites are indicated. Previously annotated and apparently novel sites are indicated.

mAb414				
Ion	Protein	Sequence	Location	Novel?
1284.54	Nup358	K.DSLTPHYGR S	T2456, S2454	Known, Known
1354.64	Nup358	R.FEISATLSEPR G	S945	Novel
1548.63	Nup358	R.FGSETTGFNFSEK S	S2270	Novel
1676.76	Nup153	K.NTSLPPLWSPFAER S	S209	Known
1959.83	Importin a2	R.NVSSPFDGATPLGENR N	S62	Known
1442.75	Nup214	R.IFWAKKQSPQAK S	S678	Known
1854.95	Nup214	R.TPISQPSLPHAAFFAK S	S1023	Novel
1311.63	Nup88	R.FFTSSTLTK.H	S168	Novel
1323.57	Nup88	R.EDVEVAESPLR.V	S517	Known
1439.72	RGPD58/7	R.YVASVLGLTSPRQ	S21	Known
Anti-Nup107				
Ion	Protein	Sequence	Location	Novel?
1698.88	Nup96	K.PAPFPQDSQPEVEQLGR.V	S834 or S932	Novel
1971.75	Nup96	K.YGLQDSDEEEHPSK.T	S858	Known
1495.88	Nup133	R.RQPLAGLPGSPTR.T	S27, T28	Novel, Novel
2128.06	Nup133	R.KGLPLGSAVSPVLFSPVGR.R	S45, S50	Known
1999.963	Nup133	K.GLPLGSAVSPVLFSPVGR.R	S45, S50	Known

Conclusions

- Immunoprecipitation afforded high-purity isolation of NPC sub-complexes from mitotic cells.
- Analysis of sub-complex constituents revealed the expected proteins and some surprises, such as abundant Myosin-9 in the Nup107-160 complex IP, and Nup133 associated with FG-domain complexes.
- Phosphopeptide analysis revealed seventeen sites of mitotic phosphorylation, eight of which were wholly novel sites.
- MALDI analysis using a MALDI LTQ Orbitrap mass spectrometer provided rapid, high mass accuracy (<5 ppm) identification of the sub-complex protein constituents and sites of phosphorylation.
- The MALDI format, in combination with the sensitive MSⁿ performance of the LTQ ion trap, allowed for manual, prolonged 'revisiting' of phosphopeptides that were not successfully identified in an automated first pass, thus permitting additional identifications.

References

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