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Identifying post-translational modifications of NEMO by tandem mass spectrometry after high affinity purification

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Abstract

An integral component of NF- κ B signalling is NEMO, NF- κ B essential modulator, a regulatory protein of the I κ B kinase (IKK) complex. Post-translational modifications of NEMO, including phosphorylation, SUMOylation, and ubiquitination are critical events during stimuli induced NF- κ B activation. Here we demonstrate a method to detect post-translational modifications of NEMO using cells stably expressing polyhistidine tagged NEMO which allows for high-affinity purification of NEMO following rapid denaturing lysis and characterization by MS/MS. We identified a previously uncharacterized basal phosphorylation of NEMO at Serine 387 and tested the biological significance of this phosphorylation through a somatic genetic complementation analysis using the NEMO mutants S387A, S388D, and P388I in 1.3E2 NEMO-deficient murine pre-B cells. NF- κ B signalling induced by bacterial lipopolysaccharide, Interleukin-1 β or the DNA damaging agent etoposide was not perturbed by these mutations of NEMO. Thus, S387 phosphorylation of NEMO is not a general requirement to mediate efficient NF- κ B signalling and therefore may have cell type and/or stimulus-specific activity in vivo.

Keywords

NEMO; NF- κ B; phosphorylation; MS/MS; polyhistidine purification

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Introduction

Nuclear Factor kappa B (NF- κ B) is a family of transcription factors responsible for regulating numerous cellular processes including innate and adaptive immunity, cell survival, and inflammation [1,2]. The NF- κ B signalling pathway is unique in the fact that a diverse group of structurally and functionally distinct molecules can induce transcription by NF- κ B, including bacterial lipopolysaccharide (LPS), tumor necrosis factor (TNF), Interleukin-1 β , as well as intranuclear events such as double strand breaks [3]. NF- κ B is normally sequestered in the cytoplasm by the inhibitor of kappa B (I κ B) protein, preventing translocation to the nucleus and subsequent gene transcription [4]. Following cell stimulation, activated I κ B kinase (IKK) complex phosphorylates I κ B proteins, ultimately leading to the degradation of I κ B and the liberation of NF- κ B. At the center of this signalling pathway is NEMO, the NF- κ B Essential Modulator [5,6]. NEMO is the key regulatory element of the IKK complex and undergoes numerous post-translational modifications (PTMs) required for NF- κ B signalling, including phosphorylation, ubiquitination, and SUMOylation [2,7-9]. Furthermore, NEMO plays a critical role in DNA damaged-induced NF- κ B signalling in a role independent of its function in the IKK complex [3,10,11]. While the biological importance of NEMO PTMs has been reported under a variety of stimuli [2,4], the detection of PTMs has numerous technical limitations. These limitations include both the transient nature of these modifications, as well as low overall abundance of modified NEMO inside the cell. Our goal was to develop a method to rapidly lyse cells under denaturing conditions to prevent loss of modified NEMO and then enrich NEMO levels to a sufficient quality and quantity to detect modifications by tandem mass spectrometry (MS/MS) and identify a previously uncharacterized NEMO modification site

Here we employ denaturing conditions using guanidine HCl to rapidly lyse cell lines stably expressing 6 $\times$ His-tagged NEMO. After purification, we identified several modified sites on NEMO utilizing nanoflow capillary chromatography coupled with high mass accuracy tandem MS. Furthermore, we investigated the biological impact of one particular site through genetic complementation experiments in a NEMO-deficient cell line.

Methods

Plasmids and Mutagenesis

For NEMO purification, an N-terminal 6 $\times$ Histidine tag was inserted in frame with NEMO in pcDNA3.1 (Invitrogen). Site directed mutagenesis for reconstitution experiments was performed via QuikChange II (Stratagene). Using pcDNA3.1 (+) NEMO wild-type as a template, NEMO S387A was generated using the following primer pairs: 5'-ctcctcggggggggccctcctctggctg-3' and 5'-cagccagaggaggggcccccccgaggag-3'. NEMO S387D was generated using 5'-ctcctcggggggggcctcctcctctggctg-3' and 5'-cagccagaggaggggaccccccgaggag-3'. NEMO P388I was generated using 5'-ccagaggaggagcatccccgaggagcca-3' and 5'-tggtcctcggggatgctcctcctctgg-3'. Custom primers were ordered from Invitrogen. All mutagenesis was verified by DNA sequencing.

His NEMO Purification

5×10^7 HEK293 cells stably expressing 6 $\times$ His NEMO (see below) were used for each purification. Cells were washed once in PBS and spun down at $400 \times g$ for 5 minutes at 4 $^{\circ}$ C. Cells were lysed in guanidinium lysis buffer (6 M guanidine HCl, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) for 10 minutes at 4 $^{\circ}$ C. Lysates were homogenized by passing through an 18 gauge needle (BD) three times and sonication (4 rounds of 10 seconds on ice). Homogenized lysates were subjected to centrifugation at $10,000 \times g$ for 15 minutes at 4 $^{\circ}$ C. Supernatant was loaded on Bio-Rad Glass Econo-

Columns (0.7 × 15 cm) containing 1.5 ml Ni-NTA Fast Flow Resin (Qiagen), which had been equilibrated in lysis buffer. Washes were conducted using a urea buffer (8 M urea, 50 mM sodium phosphate, 300 mM sodium chloride) with 5 mM imidazole (2×) and 10 mM imidazole (2×). Protein was eluted in 2 ml fractions consisting of urea buffer with 25 mM, 50 mM, or 75 mM imidazole. Fractions were then concentrated using Amicon Ultracel centrifugal filter units to approximately 200 µl (Figure 1A).

SDS-PAGE Analysis

Eluted fractions were then mixed with equal volumes of 2× SDS lysis buffer and heated at 70° C for 3 minutes. Samples were loaded on 4-12% gradient NuPAGE gels (Invitrogen) running at 100 V for 60 minutes. Gels were washed in 150 mm petri dishes in Milli-Q water for 15 minutes and subjected to coomassie staining with GelCode Safe (Pierce) for one hour. Gels were destained in Milli-Q water overnight. Care was taken when handling gels to prevent keratin contamination [5,6,12].

Tandem mass spectrometry

Bands were excised from the gel, rinsed with 100 mM ammonium bicarbonate, and dried. Cysteines were reduced using 10 mM dithiothreitol in 100 mM ammonium bicarbonate at 56°C and alkylated with 55 mM iodoacetamide. Proteins were digested overnight at room temperature with 12.5 ng/µL trypsin. The proteins were eluted from the gel by alternating four times between 50% acetonitrile, 5% formic acid and 50 mM sodium bicarbonate and an additional three times alternating between 100% acetonitrile and 50 mM NH₄CO₃. Eluted peptides were combined and dried.

Phosphopeptides were enriched via immobilized metal affinity chromatography using magnetic beads (Qiagen, Valencia, CA). Following equilibration with water, the beads were treated with 40 mM EDTA (pH 8.0) for 30 min with shaking, and washed three times with water again. The beads were then incubated with 100 mM FeCl₃ for 30 minutes with shaking and finally were washed three times with 80% acetonitrile/0.1% trifluoroacetic acid (TFA). Samples were likewise resuspended in 80% acetonitrile/0.15% TFA and incubated with beads for 45 min with shaking. The resultant mixture was washed three times with 1 ml 80% acetonitrile/0.1% TFA, and eluted using 1:1 acetonitrile/0.7% NH₄OH in water. Eluted phosphopeptides were acidified immediately with 4% formic acid and lyophilized to ~5 µl [1].

Peptides were separated using a NanoAcquity ultra high-pressure liquid chromatography system (Waters) coupled to LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a precolumn (75 µm inner diameter, packed with Magic C18 100 Å 5 µm particles, Bruker) at a flow rate of 1 µL/min. Peptides were eluted through an analytical column (75 µm inner diameter, packed with Magic C18 100 Å 5 µm particles, Bruker) using a 90-min linear gradient from 7.5% to 28% acetonitrile with 0.2% formic acid and a flow rate of 300 nL/min with monitoring of effluent by MS/MS.

For the LTQ-Orbitrap survey scan performed in the Orbitrap (MS1, resolving power = 60,000) was used to select ten precursors for tandem mass spectrometry (MS2) analysis. Selected peptides were fragmented by beam-type collision activated dissociation (HCD) (normalized collision energy = 30) and analyzed in the HCD cell Orbitrap analyzer. Precursors with unassigned or +1 charge states were not selected for MS2 analysis. Precursors were selected based on their calculated monoisotopic m/z. A small window (10 ppm) was used to exclude a maximum of 500 selected precursors for 45 s with a repeat count of 1. Automatic gain control was used at target values of 1,000,000 for MS1 analysis and 100,000 for HCD MS2 analysis.

MS/MS data were analyzed using the Coon OMSSA Proteomics Software Suite [13]. The Open Mass Spectrometry Search Algorithm was used to search spectra against a concatenated target-decoy database consisting of human protein sequences [14]. For all searches, tryptic peptides were created in silico allowing up to three missed cleavages. The precursor mass tolerance was set to 100 ppm, and the monoisotopic mass tolerance was set to ± 0.5 Da for fragments ions. Carbamidomethylation of cysteines was included as a fixed modification, and oxidation of methionines and phosphorylation of serines, threonines and tyrosines were added as a variable modification. Results from each experiment were then filtered to a 1% false discovery rate (FDR) using high-resolution FDROptimizer. The FDROptimizer conducts false discovery analysis at the peptide level using a two-dimensional analysis. One of these dimensions is OMSSA e-value, whereas the other is the precursor mass error.

Cell Culture and Generation of Stable Cell Lines

1.3E2 cells were cultured in RPMI 1640 medium (HyClone Thermo) supplemented with 10% fetal bovine serum and 0.05 mM β -mercaptoethanol, penicillin (100 IU/ml) and streptomycin (100 μ g/ml) per ml in a 5% CO₂ humidified incubator (Forma). HEK293 cells were cultured in DMEM medium (Corning Cellgro) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μ g/ml) per ml in a 10% CO₂ humidified incubator (Forma). HEK293 cells were grown on tissue culture plates (Techno Plastic Products, Switzerland) that had been treated with 0.1% gelatin. The 6 $\times$ His NEMO HEK293 stable cell line was generated via calcium phosphate transfection [15] using pcDNA3.1(+) 6 $\times$ His NEMO and selected with G418 (1 mg/ml). 1.3E2 stable pools and clones were generated via electroporation [16] and selected with G418 (1 mg/ml).

Reagents and Antibodies

Etoposide (VP-16) was purchased from Santa Cruz Biotechnology. LPS and IL-1 β was purchased from Calbiochem. Antibodies against NEMO (FL-419) and Actin (I-19) were purchased from Santa Cruz Biotechnology. OCT1 consensus oligonucleotide was purchased from Promega.

Results

NEMO Purification

HEK293 cell lines stably expressing 6 $\times$ His tagged NEMO were lysed and subjected to purification using a NI-NTA column. Surprisingly, NEMO was eluted at relatively low concentrations of imidazole compared with other proteins eluted using imidazole [17]. We found that the peak fraction contained 50 mM imidazole (Figure 1), with an approximately yield of 300 ng when compared with a BSA ladder.

MS/MS Analysis

Purified NEMO excised from the gel was enzymatically digested with trypsin and enriched for phosphopeptides using IMAC prior to LC-MS/MS analysis. During data processing, phosphorylation events were identified on four NEMO peptides; manual validation of multiple peptide spectral matches (PSMs) localized a phosphorylation site to serine 387 (Figure 2).

Functional Reconstitution

Further analysis was performed on serine 387 based on the serine-proline sequence, as well as the proximity to a region rich in prolines (residues 388 to 398) whose biological significance is a source of discussion [18,19]. To test the importance of the site in NF- κ B

signalling, we mutated residue 387 from serine to alanine as well as a phospho-mimetic residue, aspartic acid. In addition, we also mutated the proline residue at position 388 since mutation of the adjacent proline can often abrogate serine phosphorylation. pcDNA3.1 NEMO constructs containing the NEMO point mutants were electroporated in to 1.3E2 cells, a murine pre-B cell line that is deficient for NEMO and previously employed to study NEMO functions [6,20]. Due to the fact that these cells are NEMO deficient, they have an NF- κ B signalling defect, which can be rescued through reconstitution of functional NEMO. After electroporation and treatment with G418, we selected 1.3E2 clones expressing NEMO point mutants S387A, S387D, or P388I at levels approximating endogenous expression. In addition, expression amongst functionally reconstituted 1.3E2 stable pools and clones was consistent (Figure 3A and 3B). To assay functional reconstitution of NF- κ B signalling, we utilized an electrophoretic mobility shift assay (EMSA). While there are several biochemical and functional assays that can be used to monitor NF- κ B activation, EMSA is particularly valuable because it represents the key signal-proximal step in NF- κ B activation, the binding of NF- κ B to DNA [21]. It is also a robust and quantifiable technique to measure activation of NF- κ B by a variety of stimuli and is reproducible amongst different cell types and culture conditions. Furthermore, it does not require transient or stable expression of reporter genes or depend on transcription or translation of a gene product, which may also be altered by other post-translational events.

While 1.3E2 cells fail to undergo LPS- and VP-16-dependent NF- κ B activation, two independent stable pools of NEMO S387A and stable pools of S387D and P388I were able to functionally reconstitute NF- κ B activation (Figure 3A). To ensure that a subtle phenotype was not being obscured through evaluation of stable pools, we isolated individual clones that were stably reconstituting NEMO protein with the described mutations (Figure 3B). Three independent NEMO S387A clones were subjected to treatment with LPS, VP-16, and IL-1 β all of which were able to recapitulate wild-type NF- κ B signalling when compared with the parental cell line, 70Z/3, which expresses endogenous NEMO (Figure 3C and 3D). Furthermore, one representative NEMO S387A clone was subjected to a VP-16 time course and compared with 70Z/3 cells, with no apparent defect (Figure 4).

Conclusion

Observation of post-translational modifications in signalling proteins is an important step in dissecting signalling cascades. The generation of HEK293 cells stably expressing hexahistidine tagged NEMO appears to be a robust approach for discovering post-translational modifications. In this paper, we purified NEMO from unstimulated cells via nickel chelate chromatography and examined it by MS/MS analysis.

This work is particularly beneficial because of the ability to rapidly lyse cells using guanidinium. Post-translational modifications are rapidly added and removed throughout cell signalling cascades, and NEMO in particular undergoes a number of transient PTMs that are highly time sensitive [9,10]. Traditional co-immunoprecipitation techniques require native conditions that allow for endogenous proteases, phosphatases, and other enzymes that can prevent detection of post-translational modifications. In addition, rapid lysis under denaturing conditions also prevents post-lysis post-translational modifications from occurring. We feel that the technique employed here will best preserve any PTMs that might have occurred prior to cell lysis.

Using this technique, we identified Serine 387 of NEMO as a new basal phosphorylation site. We further found that NEMO S387A, S387D, or P388I mutants stably expressed in NEMO-deficient 1.3E2 cells did not perturb NF- κ B signaling induced by LPS, IL-1 β , and VP-16. Therefore, basal phosphorylation of S387 is not a functional requirement for NF- κ B

signaling in the specific cell and stimuli analyzed but could be significant in other cellular and stimuli contexts in vivo. This approach may be particularly useful to discover previously uncharacterized stimulus- and time-dependent modifications of NEMO.

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Highlights

We developed a new platform for identifying post-translational modifications of NEMO

We observed phosphorylation of NEMO residue 387

Phosphorylation of serine 387 is not an absolute requirement for NF- κ B signalling.

Figure 1.

Purification of 6× His-NEMO from HEK293 cells. (A) Purification flow-chart describing the steps for purification of 6xHis-NEMO from HEK293 cells. (B) Final elution fractions following purification. Proteins were purified from HEK293 cells stably expressing 6×His NEMO and wild-type HEK293 cells on a NiNTA column. Protein was eluted with urea base buffer containing the indicated imidazole concentration in 2 ml fractions.

A)

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1  MNRHLWKSQ LCEMVQPSGGPAADQDVLGEESPLGKPAMLHLPSEQGAPET
51  LQRCLEENQELRDAIRQSNQILRERCEELLHFQASQREEKEFLMCKFQEA
101 RKLVERLGLKLDLKRQKEQALREVEHLKRCQQQMAEDKASVKAQVTSLL
151 GELQESQSRLEAATKECQALEGRARAASEQARQLESEREALQQQHSVQVD
201 QLRMQGQSVEAALRMERQAASEEKRKLAQLQVAYHQLFQEYDNHIKSSVV
251 GSERKRGMQLEDLKQQLQQAEEALVAKQEVIDKLKEEAEQHKIVMETVPV
301 LKAQADIYKADFQAERQAREKLAEEKELLQEQLQLOREYSKCLKASCQES
351 ARIEDMRKRHVEVSQAPLPPAPAYLSSPLALPSQRRSPPEEPPDFCCPKC
401 QYQAPDMDTLQIHVMETCIE

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Figure 2.
Identification of phosphorylated residues by MS/MS. A) Primary amino acid sequence highlighting the position of Serine 387. (B) Mass spectra for the identification of the phosphorylation of Serine 387.

Figure 3.

Functional reconstitution of NEMO S387A, S387D, and P388I mutants. (A) Evaluation of 1.3E2 pools stably reconstituted with NEMO S387A (two pools), NEMO S387D, or NEMO P388I. Cells were stimulated with LPS (10 μg/ml) for 30 minutes or VP-16 (10 μM) for 120 minutes and assayed for NF-κB activity by EMSA. (B) From a stable pool, individual cells were isolated, expanded, and assayed for NEMO expression by western blot analysis using FL-419 antibody and actin (C) Three independent 1.3E2 clones expressing NEMO S387A were compared with the parental cell line, 70Z/3, for NF-κB activity after stimulation with LPS (10 μg/ml) for 30 minutes or VP-16 (10 μM) for 120 minutes. (D) Indicated cell clones were also stimulated with LPS and IL-1β (10 ng/ml) for 30 minutes.

Figure 4.

Analysis of NEMO 1.3E2 cell lines. 70Z/3 or 1.3E2 NEMO S387A cells (Clone 15D4) were stimulated with LPS (10 μ g/ml) for 30 minutes or VP-16 (10 μ M) for the time indicated. Cells were lysed and assayed by EMSA for NF- κ B and Oct activity.