FEBS Letters 580 (2006) 345–350

Characterisation of the interface between nucleophosmin (NPM) and p53: Potential role in p53 stabilisation

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Received 29 September 2005; revised 5 December 2005; accepted 8 December 2005

Available online 19 December 2005

Edited by Varda Rotter

Abstract **We have used surface plasmon resonance to quantify the kinetics and stoichiometry of the interaction between p53 and nucleophosmin (NPM). Domains characterising the interface between the two proteins were identified by chemical cross-linking, proteolytic digestion and mass spectrometry based peptide mapping.We show that the C-terminal domain of NPM (residues 242–269) interacts with two regions of p53 (residues 175–196 and residues 343–363) which belong, respectively, to the DNA binding domain and the tetramerisation domain. Potential biological consequences of such interactions are discussed.**

Keywords: Nucleophosmin; p53; Stabilisation; Interaction domains; Chemical cross-linking, peptide analysis; SELDI

1. Introduction

NPM/B23 (nucleophosmin) is one of the most abundant phosphorylated proteins in the nucleolus. A characteristic of this protein is that it is more abundant in proliferating cells whether they are normal or not. Its role during proliferation was originally believed to be restricted to ribosome maturation [1,2]. In fact, NPM is a multifunctional protein; it binds to pRb and synergistically stimulates DNA polymerase α [3] and also binds to the centrosome inhibiting duplication [4]. Nucleophosmin is furthermore involved in several transcription processes through its interaction with transcription factors and its histone chaperone activity [5–7]. NPM is induced after genotoxic stress where it allows cells to resist DNA damage either by increasing their DNA repair capacity or by decreasing the apoptotic signal [8–11]. These functions have been further related to the capacity of NPM to stabilise or inhibit p53 [12–14]. Cellular determinants regulating such opposite effects are not well understood. An important factor seems to be the level of NPM expression. NPM could interact differently with the p53-MDM2-ARF pathway according to its level of expression. This is illustrated by a recent report which shows that NPM exerts its effect on p53 stabilisation or inhibition through its interaction with ARF [15]. However at high levels, NPM can stabilise p53 even in an $ARF^{-/-}$ cell line thus suggesting a direct interaction. In this context, there are two questions that should be addressed first, what domains of p53 and NPM contribute to the interaction, and secondly, what are the effects of this interaction? In order to answer these questions, we analysed the kinetic parameters of the NPM and p53 interaction using surface plasmon resonance (SPR) technology. In parallel, by combining NPM–p53 chemical cross-linking to a surface-enhanced laser desorption and ionisation (SELDI) analysis of cross-linked peptides, we mapped their interaction domains.

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Abbreviations: ACN, aceto-nitrile; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide; GST, glutathione transferase; NHS, *N*-hydroxysuccinimide; NPM, nucleophosmin; NES, nuclear export signal; SELDI, surface-enhanced laser desorption and ionisation; SPR, surface plasmon resonance; TFA, trifluoroacetic acid

2. Material and methods

2.1. Chemicals, enzymes, proteins

Recombinant glutathione transferase (GST) and GST-p53 proteins in PBS +30% glycerol were a gift of Dr. K. Gaston (Bristol University UK). Human placenta total RNA (636527) was from BD Biosciences. AmpliTaq DNA polymerase was from Applied Biosystems. Protein A was from Calbiochem. Polyclonal antibodies antiNPM (3542) and antiGST (2622) were from CST. Protease inhibitors cocktail tablets (EDTA-free) was from Roche Applied Science. HiTRAP chelating and affinity columns were from Amersham Biosciences. All chemicals were from VWR or from Sigma and were of analytical grade.

2.2. Plasmid construction

NPM ORF was amplified using 5′GACGACAAGCATATGGAAGATTCGGATGGACA3′ and 5′GCTCCTCGAGTTAAAGAGACTTCCTCCACTGC3′, respectively, as forward and reverse primers and cDNA from Human Placenta. The forward primer contains a *Nde*I site before the translation initiation codon. The reverse primer contains a *Xho*I site after the stop codon. The NPM ORF was inserted between the *Nde*I and *Xho*I sites of the pNT1 plasmid (pET-19b plasmid without the enterokinase binding site).

2.3. Production and purification of recombinant His-tagged NPM

BL21 (DE3) bacteria were transformed with pNT1/NPM plasmid and grown in 1 l of terrific broth at 30 °C until an $OD_{600\,nm}$ of 0.3. Protein NPM synthesis was induced by addition of 1 mM of IPTG for 3 h. Cells pellets were suspended in 20 ml of lysis buffer at 4 °C (Tris 50 mM pH 7.8, NaCl 500 mM, 10% glycerol, antiprotease cocktail). Bacteria were lyzed using a French press. Bacterial debris were eliminated by centrifugation. NPM protein was purified using three different columns: a heparin sepharose column (HiTRAP Heparin HP) followed by a purification on a nickel chelating column (HiTRAP chelating HP) and an anion exchange column Mini Q PC. NPM protein purity was checked using SDS–PAGE before dialysis against T buffer (Tris 50 mM pH 7.8, 10% glycerol, 1 mM DTT). NPM was concentrated to 1–2 mg/ml using centriprep concentrators.

0014-5793/$32.00
doi:10.1016/j.febslet.2005.12.025

2.4. Binding analysis by SPR

Binding studies were performed using a Biacore 1000 surface plasmon resonance instrument (Biacore, Inc.). Protein A (100 μg/ml) in 10 mM Na acetate (pH 5.2) was covalently attached to CM5 sensor chip surfaces through its amine group [16]. Antibodies (polyclonal IgG) against NPM were then bound to the immobilised protein A. Binding of GST-p53 was studied by injecting 60 μl of GST-p53 solution at various concentrations over immobilised NPM at a flow of 10 μl/min. All experiments were performed at 25 °C.

2.5. Cross-linking analysis

GST-p53 (9 μM) was incubated at 4 °C for 60 min with NPM at various ratios of molar concentration in PBS supplemented with 30% Glycerol. Proteins were then incubated at 25 °C for 15 min before the addition of the cross-linking mixtures (5 mM *N*-hydroxysuccinimide (NHS) and 20 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC), final concentrations). After 2 min incubation at 25 °C the reaction was stopped by adding SDS–PAGE loading buffer. Reaction products were analysed on SDS–PAGE.

2.6. Peptide mapping and SELDI analysis

Non-cross-linked and cross-linked material separated by SDS–PAGE were visualised by Coomassie staining and excised into Eppendorf tubes. The bands were destained by successive washing with 150 μl aliquots of 50:50 (v/v) aceto-nitrile (ACN): 25 mM NH_4CO_3 and then dried in a speed vac. Tryptic digestion was carried out by adding 5 μl of trypsin (Sequencing grade, Promega) solution (0.1 μg/μl in 1% acetic acid) and 25 μl of 25 mM ammonium bicarbonate pH 8.0. Gel slices were incubated at 37 °C overnight. Peptides were extracted by the addition of 30 μl of 50:50 (v/v) ACN: H2O, 5% trifluoroacetic acid (TFA), incubation for 30 min and removal of the supernatant to a clean Eppendorf tube. This process was repeated twice and the pooled supernatants were dried to completion before reconstitution by the addition of 5 μl of a solution of 0.1% TFA 5% acetonitrile.

Aliquots (1 μl) of peptide solutions were applied to either SEND™ or H4 ProteinChip™ surfaces, 1 μl of an appropriate matrix solution was added for cocrystallisation and the ProteinChips™ were analysed using a SELDI™ ProteinChip™ reader. The sequences used for analysis predictions can be retreived in databanks with the following protein identification: p53 (CAA42627), NPM (AAH08495) and GST (AAA57107). The GST was assumed to make little difference to the peptide analysis of p53 apart from at the immediate N-terminal.

3. Results and discussion

3.1. SPR analysis of p53 binding to NPM

A GST-p53 fusion protein (1.4 μM) was passed across immobilised NPM on a BIAcore sensor chip and the ensuing change in refractive index at the surface measured over time (Fig. 1). The GST-p53 was retained by the surface. GST alone did not interact with the immobilised NPM (data not shown). Dissociation rate constants (k_d) obtained from the curve shown in Fig. 1 gave values of 1.35×10^{-3} s^{-1}. This rate was then used to calculate the on rate (k_a) for the association phase (k_a of 4.3×10^3 M^{-1} s^{-1}). The overall dissociation constant (K_d) is thus 3.1×10^{-7} M. Table 1 resumes rates obtained from three separate experiments giving an average K_d of 5.07×10^{-7} M calculated from the three K_d values reported in Table 1.

SPR allows relatively accurate measurements of changes in mass at a surface and this has been empirically calibrated such that a change of 1000 resonance units (RU) in the biacore configuration corresponds to a change in mass of 1 ng for a globular protein [17]. We could thus ascertain that 0.82 fmoles of GST-p53 were retained by the immobilised NPM (1.9 fmoles); this corresponds to a monomeric stoichiometry of 0.43:1 for GST-p53:NPM. The antiNPM antibodies (directed against the N-terminal domain) were constrained by a protein A interaction that should allow maximum accessibility for the NPM–antiNPM interaction. It appears that one GST-p53 monomer binds for every two immobilised NPM monomers.

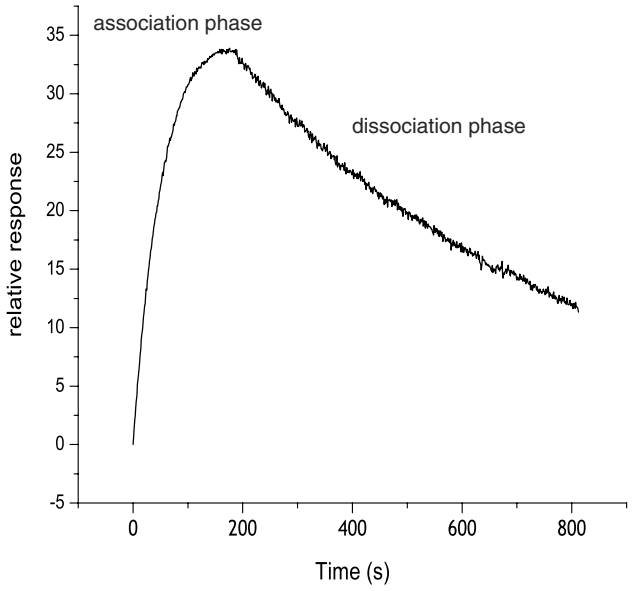

Fig. 1. Sensorgram of GST-p53 binding to immobilised NPM. 4.19×10^{-15} mole of antiNPM antibody immobilised on a protein A Biacore surface was used to capture NPM. GST-p53 (1.4 μM) was flowed across the surface at 20 μl/min. The apparent k_d was obtained by fitting the dissociation phase to the expression $R_{(t)} = R_{(o)} \exp^{(-k_d \cdot t)}$ where (R) is the relative change in resonance response as a function of time (t). The apparent k_a was obtained from $R_{(t)} = R_{(max)} (1 - \exp^{(-(k_a \cdot (C) + k_d) \cdot t)})$ at a given protein concentration of (C) where $R_{(max)}$ is the response at steady state. The apparent overall dissociation constant K_d was calculated from the ratio of k_d/k_a. The overall dissociation constant ($K_d = 3.1 \times 10^{-7}$) was calculated from the ratio of the off rates ($k_d = 1.35 \times 10^{-3}$ s^{-1}) and on rates ($k_a = 4.3 \times 10^3$ M^{-1} s^{-1}) obtained from the fit of the dissociation and association phases, respectively.

Table 1
Binding parameters for GST-p53 binding to immobilised NPM

Concentration (μM)	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (M)
1.4	4.30×10^3	1.35×10^{-3}	3.14×10^{-7}
7.0	2.14×10^3	1.94×10^{-3}	9.06×10^{-7}
7.0	8.31×10^3	2.50×10^{-3}	3.01×10^{-7}

Anti-NPM immobilised to protein A covalently bound to a CM5 Biacore surface was used to fix NPM. GST-p53 at different concentrations was then flowed across the surface.

3.2. SDS–PAGE analysis of cross-linking of NPM to GST-p53

In order to confirm the interaction between p53 and NPM we carried out carbodiimide mediated cross-linking. This technique allows the formation of zero length carboxyl-amino bonds between glutamate residues (E) or aspartate (D) residues that are in close proximity to either lysine (K) or arginine (R) residues, either in different proteins or in the same protein. Proteins that are oligomeric will cross-link at the oligomerisation interface and proteins that are intimately connected in multi protein complexes will cross-link together. The result