LC8 dynein light chain (DYNLL1) binds to the C-terminal domain of ATM-interacting protein (ATMIN/ASCIZ) and regulates its subcellular localization

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ATMIN functions. The central, highly conserved glutamine is, in many cases, flanked by either threonines (TQT) or hydrophobic residues (e.g. IQV) [7,9–15]. The DYNLL recognizing motif is generally located in disordered regions of partner proteins, frequently in the vicinity of coiled coil regions or potential dimerization domains [5,16]. As a matter of fact, structural, kinetic and thermodynamic data suggest that DYNLL might be a dimerization engine [5,7,8].

Using yeast two-hybrid techniques and DYNLL1 as bait, we retrieved ATMIN as a new interacting partner protein. ASCIZ/ATMIN (hereafter called ATMIN) share an ATM-interaction motif present near the carboxy-terminal and other peptide targets. Remarkably, co-expression of mCherry-DYNLL1 and GFP-ATMIN mutually affected intracellular protein localization. In GFP-ATMIN expressing HeLa cells DNA damage induced efficiently nuclear foci formation, which was partly impeded by the presence of mCherry-DYNLL1. Thus, our results imply a potential cellular interference between DYNLL1 and ATMIN functions.

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and ATR kinase substrate. Fine mapping studies revealed that several DYNLL1 dimers might associate with the full-length ATMIN. We describe herein this interaction and discuss its possible biological significance.

2. Materials and methods

2.1. Yeast two-hybrid screen, β-galactosidase assay, pepscan and expression of DYNLL1 and ATMIN in Escherichia coli

We have followed our own published protocols [7,10,12,15,18]. Residues 378–823 and 475–823 of ATMIN were cloned in a pET-15b vector using NdeI and EcoRI and expressed in frame with a hexa-His tag in bacteria. Expression was performed at 25 °C and purification involved binding to an affinity Ni–NTA resin followed by gel filtration using a Serva D-6900 Polyol Si300 column.

2.2. Chemical crosslinking

ATMIN fragment (378–823) and DYNLL1 were dialyzed against PBS buffer (pH 7.3). 20-fold molar excess of dimethyl pimelimidate-2 HCl (DMP) was added to the proteins. After the reaction 2 × SDS running buffer supplied by 3-mercaptoethanol was added to the samples and analyzed by tricine or glycine SDS gel electrophoresis.

2.3. NMR experiments and sample preparation

Two N15-labelled DYNLL1 samples of 109 and 85 μM concentration were prepared in aqueous solutions of 90% H2O/10% D2O in 100 mM potassium phosphate buffer, pH 7.0, 1 mM DTT. Concentrated solutions of ATMIN peptides 488-GVSRETQTSGIE-499 and 666-ESLDIETQTDFL-677 were prepared in the same buffer with estimated concentrations of 8.8 and 4.0 mM, respectively. Series of N15-HSQC spectra were recorded at 25 °C at the different steps of the titration experiments in a Bruker Avance 800 NMR spectrometer (Bruker, Rheinstetten, Germany) equipped with a z-gradient cryoprobe as previously reported [14,19].

2.4. Gel-filtration chromatography

ATMIN (378–823) fragment was analyzed by gel-filtration using Serva Polyol Si-300 column. The measurements were performed in 20 mM phosphate buffer (pH 7.3) containing 200 mM NaCl. The flow rate was 0.5 ml per minute. The hydrodynamic size was determined using Bio-Rad Gel-Filtration Standard.

The figures are not described as they are not included in the text.
2.5. Isothermal titration calorimetry

Isothermal titration calorimetry was carried out at 25 °C in PBS supplied by 3 mM 2-mercaptoethanol (pH 7.4) using a Microcal VP-ITC apparatus. 5 μM ATMIN (475–823) fragment was titrated with 660 μM DYNLL1 up to 15-fold excess of ligand with 900 s time intervals between injections. The first 2 μl injectant was removed. The measured heat changes were corrected for dilution effects using data from similar control experiments. The Origin 5.0 software package (OriginLab) for ITC was used for data processing. The simplest one site binding model (A + B = AB) was fitted to the data.

2.6. Cell transfection, immunoprecipitation and laser confocal microscopy

The coding sequence of DYNLL1 was amplified and cloned in pmCherry-C1 vector using EcoRI and SalI restriction enzymes. GFP-ATMIN was a generous gift of Dr. Behrens (Cancer Research Institute, London). COS7 cells growth, transfection, immunoprecipitation and immunofluorescence were essentially done as previously described [20].

3. Results

3.1. Identification of ATMIN as a new binding partner of DYNLL1

We have screened a human heart cDNA library using DYNLL1 as a bait protein in a yeast two-hybrid system to find new potential partner proteins. By sequence analysis of 150 positive clones we have identified ATMIN as a DYNLL1 interacting protein. Residues 362–823 of ATMIN (corresponding to the carboxy-terminal half of the molecule) were retrieved as a DYNLL1-binding region, a fragment that includes part of the core region as well as 11 SQ/TQ motifs (Fig. 1A). Most of this part of ATMIN displayed long stretches of potentially disordered regions according to the PONDR software [21] (data not shown). Interestingly, the DYNLL1-interacting motifs very frequently have sequences that include SQQ or TQT triads and are located in disordered regions of protein partners [7,11,12]. This observation raises the possibility that the DYNLL1 binding sites could match with ATM/ATR phosphorylation sites. With that in mind, four additional ATMIN fragments covering residues 362–572, 467–572, 572–823 and 691–823 were confronted to DYNLL1 in a yeast two-hybrid screen, and all of them rendered a positive interaction (Fig. 1A). Not only growth was achieved in the absence of histidine but also a clear β-galactosidase positive reaction was observed (Fig. 1A). This means that all these four constructs include SQ/TQ motifs that bind to DYNLL1 and indicates that very likely multiple DYNLL1-binding sites are present within the ATMIN sequence.

In agreement with our previous yeast two-hybrid fine mapping result we obtained 11 positive DYNLL1 binding sites using a pepscan approach (Fig. 1B), all of them with a Gln residue, frequently flanked by Thr residues. In fact, all previously predicted sequences had positive hits. Next, we inspected if these DYNLL1-binding motifs within the ATMIN sequence were indeed conserved among various mammalian ATMIN sequences. As shown in Fig. 1B, sequence alignment using the Clustal software revealed that these SQ/TQ motifs are extremely conserved, hence reinforcing a biological significance.

In order to evaluate if ATMIN and DYNLL1 bind to each other in mammalian cells, co-immunoprecipitation experiments were performed using anti-DYNLL1 antibodies and analyzing the presence of ATMIN in the immunoprecipitated fraction. As shown in Fig. 1C, anti-DYNLL1 antibodies, but not a pre-immune serum,
can immunoprecipitate ATMIN, hence indicating that the two proteins interact in a physiological environment.

3.2. Recombinant expression and characterization of ATMIN (378–823)

Next, we produced a recombinant ATMIN fragment consisting of residues 378–823 of ATMIN (corresponding to the domain enriched in SQ/TQ motifs) in E. coli which migrated in a SDS–PAGE gel at approximately 66 kDa, whereas its theoretical molecular weight is ~50.9 kDa (Fig. 2A). This is usually indicative of an intrinsically disordered protein in solution [21]. We next decided to analyze if ATMIN (378–823) was an oligomeric protein. Treatment of ATMIN (378–823) with crosslinking agents for 0, 15 or 30 min rendered no oligomers according to SDS–PAGE analysis (Fig. 2A, middle panel). On the other hand, under identical treatment conditions, the crosslinked protomers of DYNLL1, which are always in dimeric form, could be clearly observed (Fig. 2B, right panel).

When analyzed using gel filtration (Fig. 2B) ATMIN (378–823) appeared at approximately 6.5 ml, corresponding to 363 kDa when its elution volume was interpolated in a calibration curve (data not shown). Consequently, at this point we considered that ATMIN is able to adopt a non-globular or disordered conformation responsible for its apparent large molecular weight. Addition of a five molar excess of recombinant DYNLL1 to recombinant purified ATMIN (378–823) resulted in no displacement to larger apparent molecular masses in gel filtration experiments (Fig. 2B), although a clear concomitant decrease in the amount of free DYNLL1 could be observed. In addition, SDS–PAGE revealed that DYNLL1 bound to the ATMIN (378–823) fractions that eluted at ~6.5 ml (data not shown). This further proves DYNLL1 binding to ATMIN (378–823) and is also indicative that this binding does not induce a significant change in the hydrodynamic behavior of the later.

Using isothermal titration calorimetry (Fig. 2C), we could detect approximately five DYNLL1 binding sites within the ATMIN (378–823) molecule with an average $K_d$ value of 1.6 μM (assuming independent binding sites). The interaction was enthalpy driven ($\Delta H = 44.6$ kJ mol$^{-1}$) and entropically disfavored ($-T\Delta S = 11.2$ kJ mol$^{-1}$) which is in good agreement with the thermodynamic properties of other DYNLL1-binding motifs containing a TQT sequence [7,22]. This result raises the possibility that not all of the eleven binding sites that we identified using the pepscan technique could be occupied simultaneously in ATMIN (378–823), perhaps because they could not be accessible to DYNLL1.

3.3. NMR monitored titrations of DYNLL1 with two ATMIN peptides

In order to obtain structural information about the interaction between ATMIN and DYNLL1 we selected two binding regions (488-GVSRETQTSGIE-499 and 666-ESLDIETQTDFL-677) of ATMIN previously identified in yeast two-hybrid and pepscan analysis and tested the binding by NMR. The assignment of the DYNLL1 HN resonances (25 °C, pH 7.0) was based on previous publications [11,23]. The interaction of DYNLL1 with these ATMIN peptides was tested by NMR-monitored titrations of the $^{15}$N-labelled protein with the unlabelled peptides. Series of $^{15}$N-HSQC spectra were recorded for DYNLL1 at increasing amounts of both peptides as described in Materials and Methods. A large set of resonances, that includes residues known to directly contact the target peptides in previously reported complex structures [9,11] (residues in helix α2 and strand β3 of adjacent monomers of the dimer), shifts their positions upon complex formation corroborating the direct interaction. Most of these residues are common to both titrations (Fig. 3A) firmly suggesting that the peptides occupy a similar interacting interface in the complex and that they share the canonical binding mode. On the basis of the previous titration experiments...
we selected one of the ATMIN peptides (ESLDIETQTDLF) to model the structure of the complex with DYNLL1 since, as mentioned above, a large overall similitude is expected for the complexes with both tested peptides. Superposition of 15 conformers from the best cluster that defines the family of structures of the DYNLL1–ATMIN ESLDIETQTDLF peptide complex is shown in Fig. 3B. The Haddock score for this cluster is −129.2, the electrostatic contribution to the energy −378 kcal/mol, and the buried surface area 1573 Å². The orientation of the peptide is well-defined with an RMSD value of 0.65 Å for the backbone, and 1.11 Å when the heavy atoms are included. Similarly to other complexes [7,8,15], the peptide runs parallel to the DYNLL1 binding groove extending the central β-sheet with an additional sixth strand establishing characteristic H-bond networks.

3.4. Subcellular localization of DYNLL1 and ATMIN: DYNLL1 interference of ATMIN-dependent foci formation

In order to analyze the interactions of DYNLL1 and ATMIN within a cellular environment, mCherry-DYNLL1 and GFP-ATMIN plasmids were transfected in COS7 cells and the subcellular localization was analyzed using laser confocal microscopy. mCherry-DYNLL1 stained both the cell cytoplasm and the nucleus (Fig. 4A, top). In agreement with previous data [24], most of the GFP-ATMIN labelled the cell nuclei in COS7 cells (Fig. 4A, bottom). Interestingly, when mCherry-DYNLL1 and GFP-ATMIN were cotransfected in the same cell the subcellular staining was highly dependent on the amount of expressed protein. In this regard, if large amounts of mCherry-DYNLL1 were expressed in the cell, the GFP-ATMIN fluorescence was recruited to the cytoplasm (Fig. 4B, arrow).

Treatment of cells with low concentrations of the DNA methylating agent MMS is known to induce the formation of nuclear foci in which ATMIN and Rad51 colocalize [17,24]. When we treated COS7 cells with MMS and analysed the subcellular localization of mCherry-DYNLL1 and GFP-ATMIN in cells that expressed both proteins we observed that DYNLL1 binding to ATMIN did not impede completely GFP-ATMIN foci formation, and mCherry-DYNLL1 staining could be also observed in the nuclear foci (Fig. 4C). In order to quantify the effect of DYNLL1 on foci formation COS7 cells were cotransfected with GFP-ATMIN and/or mCherry-DYNLL1 and treated with 0.02% MMS for 2 h. When a monolayer of cells was transfected with GFP-ATMIN alone, 2 h of MMS treatment proved enough to induce the formation of foci in every single transfected cell. However, when GFP-ATMIN and mCherry-DYNLL1 were cotransfected, only 55% of the cells (n = 367) could form foci after MMS treatment (Fig. 4C). Among these foci-forming cells, however, only 16% showed colocalization of both proteins at the nuclear foci whereas the remaining cells had foci with only GFP-ATMIN present. Thus, these results further emphasize that binding of DYNLL1 to ATMIN clearly interferes with foci formation.

4. Discussion

In this manuscript, after screening of a human heart cDNA library with DYNLL1 as bait, we retrieved ATMIN, a recently discovered protein, as a strong interacting polypeptide. Interestingly, ATMIN had been predicted as a highly probable interacting partner
of DYNLL [7]. ATMIN is a DNA damage response protein located in the cell nucleus that colocalizes with the recombinein Rad51, though it does not interact directly with it [17,24]. ATM kinase is believed to respond primarily to DNA double strand breaks such as those caused by ionizing radiation whereas ATR kinase is believed to be mostly activated by single-stranded DNA gaps [25]. Once activated, both kinases phosphorylate several hundred effector proteins, in many cases in SQ or TQ motifs [26]. It is assumed that these linear SQ/TQ motifs are generally located in intrinsically disordered regions of these effector molecules [17,27]. Since the SQ/TQ repeats are hallmarks of DNA damage response proteins and potential substrates for the checkpoint kinases ATM and ATR [28] the behavior of ATMIN/ASCIZ was analyzed under conditions of methylating DNA damage.

The simultaneous binding of multiple DYNLL1 or multiple DYNLL2 units to the same polypeptide chain is not without precedents. For instance, the DID domain of Nup159 displays six consecutive binding sites for Dyn2 (the yeast dynein light chain), five of which also bind in a peptscan analysis and in solution [29]. In addition, two units of DYNLL1 or DYNLL2 can bind to two tandem motifs in Gkap1 [12,30]. Using peptscan approaches, we have also shown previously that other such proteins, such as human adenovirus-associated BS69 protein or human herpesvirus U19 display tandem binding sites for DYNLL1 [10]. Finally, p53BP1 displays two sequential DYNLL1 binding motifs [31].

Whether all of these 11 sites are simultaneously occupied alongside the ATMIN sequence remains to be established. In transfected cells DYNLL1 binding to ATMIN also interferes with foci formation after MMS treatment. It is then conceivable that ATMIN phosphorylation by ATM/ATR kinases under conditions of DNA damage might occur at SQ/TQ motifs that serve simultaneously as kinase phosphorylation spots and DYNLL1 binding sites. Therefore, our results indicate that DYNLL1 binding to certain cellular targets interferes with ATM/ATR phosphorylation and vice versa. Moreover, this reciprocal regulation could be present in multiple cellular proteins.

Acknowledgments

This work was supported by Grants MICINN BFU2009-10442 (I.R.C.), Consolidador-centrosoma 3D (CSD2006-00023) (M.B.), CTQ2008-0080 (M.B.), OTKA N81950 (L.N.), K81934 (K.S.), TÁMOP 42.1.1-08/09/KMR-2010-0003. We would like to thank Fernando Roncal for synthesis of the pepscan cellulose membranes. We are thankful to András Patthy for preliminary EM experiments and in-house for his generous gift of the ATMIN plasmid and Dr. József Kardos for the behavior of ATMIN/ASCIZ was analyzed under conditions of methylating DNA damage.

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