

Isolation of human NuMA protein

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Abstract NuMA is a protein involved in maintenance of nuclear structure and in the assembly of the mitotic spindle. Expression of amino-terminal deletion mutants results in a phenotype identical to that caused by a temperature-sensitive defect of RCC1 (regulator of chromosome condensation). Here we describe the isolation of NuMA protein from HeLa cells under mild conditions as a prerequisite to study its interactions with elements of the RCC1-Ran regulatory pathway. In an overlay assay, NuMA did not bind Ran·[γ -³²P]GTP. Thus it is clearly different from Ran·GTP binding proteins of similar M_r .

Key words: Chromatin condensation; Centrosome; RCC1; Ran; RanBP2; Cell cycle regulation

1. Introduction

NuMA is a 236 kDa protein found dispersed throughout the nucleus of mammalian cells during interphase and, during mitosis, associated with the pericentrosomal regions of the mitotic spindle. It had also been designated centrophilin, SPN [2], SPN [3], SP-H antigen [4], or 1F1/1H1 antigen [5]. Injection of monoclonal antibodies to NuMA before anaphase inhibits mitosis [3,6,7] and leads to formation of micronuclei [8]. NuMA appears to be involved in the nucleation and organization of mitotic, but not interphase, microtubules [9], and in spindle pole function [7], possibly by acting as a mitotic microtubule-associated protein [10,11]. It has been found to co-precipitate in immune complexes with antibodies to small nuclear ribonucleoproteins, and associates with in vitro-reconstituted splicing complexes [12].

Its primary structure, translated from the cDNA sequence, indicates a large central coiled-coil region, flanked by globular head and tail domains [5,9,11,13]. Alternative splicing of the mRNA transcript leads to proteins which differ at their carboxyl-terminal region [11,13], that carries signals for nuclear localization and association with the mitotic spindle [14]. Ectopic expression of amino-terminally deleted NuMA constructs results in failure to undergo cytokinesis and the appearance of multiple micronuclei [15]. Morphologically, this phenotype is very similar to that resulting from a temperature-sensitive mutation in RCC1, the regulator of chromosome condensation [16]. Over-expression of wild-type NuMA results in partial suppression of the terminal phenotype of temperature-sensitive RCC1 [15].

Therefore, NuMA may be a target of the RCC1-Ran pathway, which has been implicated in nuclear import of proteins [17,18], pre-mRNA processing and export [19], regulation of the initiation of S phase and chromosomal DNA synthesis [20], maintenance of nuclear structure, and the onset of and exit from mitosis [16,21]. To date, four components of this pathway have been characterized biochemically: RCC1 [22], Ran [23], RanGAP1 [24], and RanBP1 [25,26]. Here we report the isolation of NuMA from HeLa cells under mild conditions and

initial experiments to study the putative interaction with the RCC1-Ran signal pathway.

2. Materials and methods

2.1. Cell culture and synchronization of HeLa cells in suspension

HeLa S3 cells were grown in Joklik's modified MEM supplemented with 5% newborn calf serum (Biochrom, Berlin) to a density of 4×10^5 cells/ml. For synchronization in mitosis, nocodazole was added to a final concentration of 80 ng/ml. The cells were incubated for 16 h, collected by centrifugation at $800 \times g$, washed twice in PBS and once in PBS containing protease inhibitors [22], and processed immediately without freezing.

2.2. Purification of NuMA protein from HeLa cell lysates

40 ml of freshly harvested packed HeLa cells were suspended in 100 ml of lysis buffer (20 mM bis-Tris propane-HCl, pH 6.9, 1 mM EDTA, 1 mM DTT and protease inhibitors), swollen on ice for 30 min, homogenized by 8 strokes in a Dounce homogenizer, and centrifuged at $70,000 \times g$ for 45 min. In lysates of interphase cells the antigen was found in the pellet, whereas in nocodazole-blocked cells the majority was in the supernatant (not shown). NuMA from unsynchronized cells was solubilized by resuspending the pellet in lysis buffer containing 400 mM NaCl to a final volume of 100 ml with 20 strokes in the homogenizer, followed by centrifugation at $70,000 \times g$ for 30 min. The supernatant was diluted with lysis buffer to a final concentration of 100 mM NaCl, filtered through a G4 Buchner funnel and chromatographed on DMAE-Fractogel (Merck; 13.5×2.5 cm) in 20 mM bis-Tris propane-HCl, pH 6.9, 1 mM EDTA, 1 mM DTT with a flow rate of 5 ml/min and a gradient of 100 mM to 1 M NaCl. Protease inhibitors were re-added to NuMA-positive fractions. To remove residual nucleic acids, the sample was incubated with 25 U/ml of benzonase (Merck) for 2 h at 4°C. It was then gel-filtrated on a S-500 Sephacryl column (Pharmacia; 110×2.5 cm) in 25 mM Tris, pH 7.2, 25 mM NaCl, 1 mM DTT and 1 mM EDTA, at a flow rate of 0.7 ml/min. Fractions containing NuMA were applied to Lichrospher 4000 TMAE (Merck; 4×1 cm), and final purification was achieved with a gradient from 30 mM to 250 mM NaCl at a flow rate of 1 ml/min. Speed was essential during this separation to minimize proteolytic degradation, the procedure taking about 32 h. All steps were performed at 4°C.

2.3. Immunoblotting and overlay assay of renatured blots with Ran·[γ -³²P]GTP

Blots were performed as previously described [23]. Immunostaining was done with 1:500 dilutions of human autoimmune serum 2642 to NuMA, and with autoantibody affinity-purified on the 236 kDa band as described [22]. For confirmation of the identity, monoclonal antibodies SPN7 and PON2 to NuMA were utilized (kindly provided by M. Osborn, Göttingen). Secondary goat anti-human peroxidase-labeled antibodies (Amersham) were detected by incubation with a solution

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containing 0.5 mg/ml of diaminobenzidine, 0.02% CoCl_2 , and 1 $\mu\text{l}/10$ ml of 30% H_2O_2 in TBS, which was added immediately before use.

For the overlay assay, 50 μg of soluble HeLa protein fraction [23] per lane were separated on a 8% SDS-gel and transferred to nitrocellulose. Blotted proteins were denatured with 6 M guanidinium chloride in 50 mM Tris-HCl, pH 7.6, 1 mM DTT and renatured by dropwise addition of TBS (10 mM Tris-HCl, pH 7.6, 150 mM NaCl), 5 mM MgCl_2 , 1 mM DTT. After three 1:10 dilution steps nitrocellulose strips were blocked for 30 min with 5% skimmed milk powder in TBS- Mg^{2+} -DTT. Preincubation was done for 10 min with 3 ml TBS- Mg^{2+} -DTT containing 0.5% milk powder and 100 μM each of GTP and GDP. After incubating for 10 min with 2 nM Ran·[γ - ^{32}P]GTP, the strips were washed ten times with TBS- Mg^{2+} -DTT, dried and autoradiographed.

3. Results

3.1. Distribution of NuMA antigen in HeLa cells in interphase and mitosis

From our collection of some 5000 human autoimmune sera, 11 sera derived from patients with rheumatoid arthritis, primary Sjögren syndrome, and silicosis, recognized a 236 kDa protein band on immunoblots. In indirect immunofluorescence studies in HeLa cells the antigen showed a distribution corresponding to patterns described previously for NuMA [1], centrophilin [2], SPN [3], and SP-H antigen [11]. In interphase cells, the antigen is diffusely distributed in the nucleus whilst nucleoli remain unstained. In early mitotic stages it is enriched in the polar regions of the spindle (data not shown). The immunofluorescence pattern and the identity of the purified 236 kDa protein on immunoblots with NuMA were confirmed with monoclonal SPN7 and PON2 antibodies to NuMA, kindly provided by Dr. Osborn [3].

3.2. Purification of NuMA protein from HeLa lysates

A band of antigen was seen at about 236 kDa on immunoblots of HeLa cell lysates that were stained with human autoimmune serum 2642. In most fractions one or two bands

of lower molecular weight (about 190 kDa) were also observed, even when cells were immediately boiled in electrophoresis buffer. These bands could represent isoforms of NuMA derived from differential splicing of transcripts [13,14]. Human autoantibody 2642, affinity purified from either band, re-stained both bands. PON2 and SPN7 monoclonal antibodies stained the 236 kDa band only. In addition, all of our 11 autoimmune sera with a NuMA-like distribution pattern also stained the 236 kDa and the 190 kDa band of NuMA protein on blots (not shown).

In mitotic cells treated with nocodazole (or colchicine, vinblastine, taxol) most of the antigen is readily soluble in low salt buffers, whereas higher concentrations of sodium chloride were required to extract it from the nuclear pellet of untreated, unsynchronized cells. This may reflect its association with other molecules in the nuclei of interphase cells, because, once extracted, the antigen is soluble under low salt conditions.

NuMA has been isolated previously from antibody affinity columns upon denaturation with guanidinium chloride [27], and from immunoprecipitates after denaturation by SDS-gel electrophoresis [8,27]. As a prerequisite for studying the possible association of native NuMA with other proteins, we developed a rapid purification scheme applying mild conditions more likely to preserve native structure and function (Fig. 1). In this procedure, the pellets of HeLa cell lysates were extracted with 400 mM NaCl, and the extract was chromatographed on DMAE Fractogel (Fig. 1A).

Upon gel-filtration of these fractions on Sephacryl S-500, NuMA from both interphase and mitotic cells eluted with an apparent M_r of about 1.5×10^6 , suggestive of oligomer formation. Fig. 1B shows this for NuMA from unsynchronized cells. These fractions were also tested on immunoblots with antisera to RCC1 and Ran to detect co-enrichment of these proteins. The results were negative and are not shown.

Final purification was achieved on TMAE Lichrospher

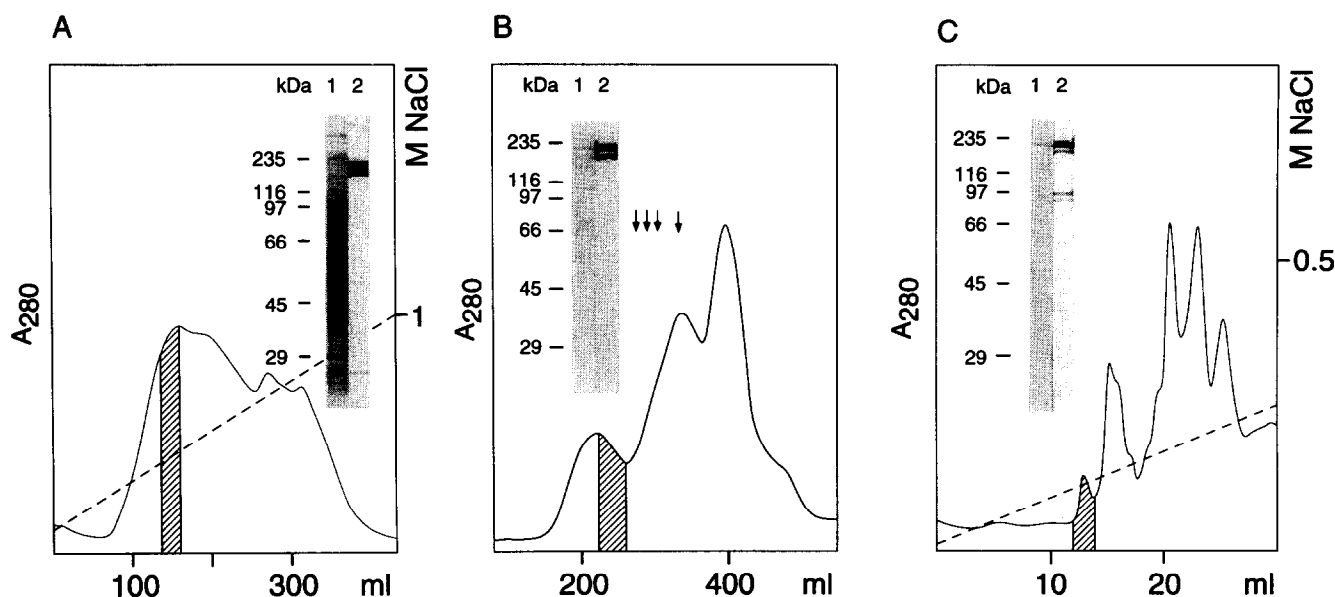


Fig. 1. Purification of NuMA protein from HeLa cells. (A) Chromatography of NuMA protein from cell lysates on DMAE-Fractogel in 20 mM bis-Tris propane-HCl, pH 6.9. (B) Gel-filtration on Sephacryl S-500 in 25 mM Tris-HCl, pH 7.2. The markers indicated by arrows were, from left-to-right (M_r : thyroglobulin (669,000); ferritin (440,000); β -amylase (200,000); bovine serum albumin (66,000). (C) Chromatography on Lichrospher TMAE 4000 in 25 mM Tris-HCl, pH 7.2. Insets: 1, blot with Ponceau S after gel-electrophoresis and transfer to nitrocellulose of hatched antigen fractions; 2, immunostaining of blotted proteins with human autoimmune serum 2642.

(Fig. 1C). This resulted in a 199-fold enrichment of the NuMA protein (Table 1), which still contained traces of antigen with higher electrophoretic mobility. We calculated the number of NuMA molecules per cell to be close to one million, compared to 10 million of Ran and about 400,000 of RCC1. This protein, purified under mild conditions, will be used to study the putative interactions with the known elements of the RCC1-Ran pathway.

3.3. Overlay assay with Ran·[γ -³²P]GTP to correlate NuMA with RanBP2 or other Ran·GTP binding proteins

Of the elements of the RCC1-Ran pathway so far identified, a protein tentatively designated RanBP2 has an apparent molecular weight of <230,000. RanBP2 selectively binds Ran·GTP but not Ran·GDP (Bischoff et al., in preparation). It is therefore considered an effector of the pathway. Persistence of its binding to Ran·GTP in the presence of EDTA with an excess of unlabeled GTP indicates that it stabilizes GTP binding to Ran. Other bands of similar mobility in gel-electrophoresis have been identified by their capacity to bind selectively to Ran·GTP [26]. On binding to various targets, RanBP2 bound to Ran·GTP might help to fulfill one of the functions which are disturbed by the loss of RCC1 in various systems. The amino acid sequence of RanBP2 is unknown. Because of the similar M_r of this protein to that of NuMA and the putative involvement of NuMA in the RCC1-Ran pathway discussed below, it was suspected that NuMA could be RanBP2 or one of the other Ran·GTP binding proteins of high molecular weight. We tested this possibility using an overlay assay with Ran·[γ -³²P]GTP on renatured blots (Fig. 2). Ran-binding activity of a corresponding mass is present in the crude HeLa lysate. However, it is not co-enriched but lost on purification of NuMA. Thus, NuMA is clearly different from the Ran-binding proteins so far described.

4. Discussion

NuMA is essential for the establishment of mitotic spindles [7,8] and the re-formation of the nucleus after mitosis [15]. In this study we present a rapid purification procedure for NuMA, since the purified protein is required for biochemical investigation of its function. NuMA isolated from cells which were blocked in mitosis by nocodazole was readily soluble in buffers

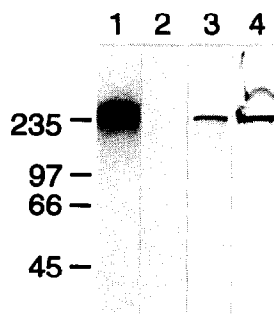


Fig. 2. Overlay assay of blots containing NuMA and RanBP2 with Ran·[γ -³²P]GTP. Lanes 1 and 3, soluble fraction of lysate of nocodazole arrested mitotic HeLa; lanes 2 and 4, purified NuMA; lanes 1 and 2, overlay assay with Ran·[γ -³²P]-GTP after renaturation; lanes 3 and 4, immunostaining with human autoimmune serum 2642 to NuMA.

Table 1
Enrichment of NuMA protein

Fraction	Total protein (mg)	NuMA (mg)	Yield (%)	Enrichment factor
70,000 × g pellet	1510	7.6	100	
400 mM salt extract	705	3.6	47	1.01
Fractogel DMAE 650	20	0.47	6.3	4.7
Sephacryl S-500	2.7	0.26	3.4	19
Lichrospher TMAE 4000	0.03	0.03	0.4	199

Values refer to 50 ml of packed HeLa cells ($\approx 2 \times 10^{10}$ cells) as starting material. NuMA was quantitated by staining of gels with Coomassie blue (not shown) and of immunoblots with human autoimmune serum to NuMA.

of low ionic strength, in contrast to material from unsynchronized cells. In the purification steps NuMA from both sources behaved similarly, except that the final preparation from mitotic material still contained other proteins. Hence the purification from nocodazole-blocked cells is not shown.

The experiments of Compton and Cleveland [15] reveal the possibility of NuMA being involved in cell cycle regulation through the RCC1-Ran pathway: expression of N-terminally deleted NuMA results in a terminal phenotype with cells that fail to undergo cytokinesis and assemble micronuclei. Morphologically they are very similar to the temperature-sensitive RCC1-defective tsBN2 cells, derived from golden hamster BHK21 cells [16]. Over-expression of wild-type NuMA in tsBN2 cells partially suppresses this phenotype by restoring post-mitotic assembly of a single normal-sized nucleus.

The RCC1 gene product was shown to be bound to chromatin and to exchange guanine nucleotide bound to the Ras-related nuclear protein Ran [22]. The RCC1-Ran pathway has been implicated in regulating the initiation of DNA synthesis [20], activation of the cdc2 kinase and induction of mitotic events [17,28,29], in maintenance of nuclear structure [29], RNA processing and export [19], and import of proteins into the nucleus [17,18].

So far, the targets of this pathway are unknown. They may include Ran-binding proteins, which recognize only its activated, GTP-bound form [25,26]. NuMA may also be a downstream element, considering the merely partial complementation of the RCC1-defective phenotype by over-expression of NuMA [15]. Yet neither RCC1, Ran, nor RanBP2 co-purified with NuMA, as judged from immunoblots and from an overlay assay for proteins that selectively recognize Ran in its GTP-bound form. Larger quantities of purified native and recombinant NuMA will be required to study its putative interference with biochemical reactions of the RCC1-Ran pathway. The rapid NuMA purification scheme described here should be a step towards these goals.

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