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Biochimica et Biophysica Acta 1763 (2006) 272–281
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Nek1 shares structural and functional similarities with NIMA kinase

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Received 16 June 2005; received in revised form 12 January 2006; accepted 17 January 2006
Available online 21 February 2006

Abstract

The *Aspergillus* NIMA serine/threonine kinase plays a pivotal role in controlling entrance into mitosis. A major function attributed to NIMA is the induction of chromatin condensation. We show here that the founder murine NIMA-related kinase, Nek1, is larger than previously reported, and that the full-length protein conserves the structural hallmarks of NIMA. Even though Nek1 bears two classical nuclear localization signals (NLS), the endogenous protein localizes to the cytoplasm. Ectopic overexpression of various Nek1 constructs suggests that the C-terminus of Nek1 bears cytoplasmic localization signal(s). Overexpression of nuclear constructs of Nek1 resulted in abnormal chromatin condensation, with the DNA mainly confined to the periphery of the nucleus. Advanced condensation phenotype was associated with nuclear pore complex dispersal. The condensation was not accompanied by up-regulation of mitotic or apoptotic markers. A similar phenotype has been described following NIMA overexpression, strengthening the notion that the mammalian Nek1 kinase has functional similarity to NIMA.

Keywords: NIMA; Nek kinase; Chromatin condensation; Cell cycle; Sub-cellular localization

1. Introduction

The *Aspergillus* NIMA kinase, is required by this fungus for entry into mitosis [1,2]. As implied by its name (never in mitosis A), in the conditional absence of NIMA activity, fungal cells cannot enter mitosis and they are arrested in G_2, exhibiting interphase microtubules and uncondensed chromosomes [3,4]. The mitotic activation of NIMA is probably achieved through autophosphorylation, followed by hyperphosphorylation by Cdc2 [5]. Overexpression of NIMA results in premature chromatin condensation from any phase of the cell cycle; this effect is independent of Cdc2 activation. Consistent with this phenotype, it has been demonstrated that overexpression of NIMA in *Aspergillus* triggers histone H3 phosphorylation on Ser-10, a phosphorylation event connected with mitotic chromatin condensation [6]. An additional documented task of NIMA is the nuclear localization of cyclin B [7], possibly by phosphorylation of nuclear pore complex (NPC) proteins [8]. NIMA is localized to the NPC during early mitosis, and is essential for NPC proteins dispersal [9].

Several observations suggest that the NIMA pathway is also evolutionarily conserved. Thus, overexpression of the fungal NIMA kinase in *Xenopus* oocytes induces germinal vesicle breakdown, and overexpression in human HeLa cells results in premature chromatin condensation without mitotic spindle formation [2,10]. In addition, similar to the phenotype in *Aspergillus*, overexpression of dominant-negative variants of NIMA in human cells results in characteristic G_2 arrest [2]. Finally, mammalian genomes encode for a family of NIMA-related kinases (designated Nek1 to Nek11), which are natural candidates for performing NIMA-like mitotic activities, and recent data suggests several common characteristics [11]. Among the mammalian Nek proteins, Nek2 has the greatest sequence similarity, within the kinase domain, to NIMA. Its major activities are associated with centrosomal, spindle and kinetochore-related functions [12,13]. Nek2 has also been shown to be localized to condensing meiotic chromosomes [14], and to be involved in meiotic chromatin condensation, probably by interacting with, and phosphorylation of, the architectural chromatin protein, HMGA2 [15,16]. Recently, mitotic roles have been demonstrated for another Nek member, Nek9/Nercc1, and for its interacting proteins, Nek6 and Nek7

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doi:10.1016/j.bbamcr.2006.01.009

[17–20]. Involvement of Nercc1/Nek9 in interphase and S-phase progression was suggested by virtue of Nek9 binding to FACT, a nuclear complex active in regulation of the chromatin in relation to DNA replication and transcription [21]. Taken together, the mammalian Nek kinases have been shown to be involved in various aspects of cell cycle control, but there is no evidence for their direct involvement in chromatin condensation.

Inherited mutations in the *nek1* gene in mice result in several developmental defects. The mutants suffer from dwarfism and early mortality. Animals that survive to adulthood are either sterile (males) or subfertile (females), and develop polycystic kidneys [22]. Even though Nek1 was the first mammalian NIMA-related gene to be cloned [23], its cellular or molecular functions are unknown. We report here that Nek1 has greater structural similarity to NIMA than previously reported. Overexpression of truncated Nek1 variants induces chromatin condensation resembling the phenotype induced by NIMA overexpression in vertebrate cells.

2. Materials and methods

2.1. Plasmid construction

For construction of Nek1 expression vectors, we used the Nek1–not12 fragment [23]. A unique *Sal*I site was added by adaptor ligation just upstream to the initiator Met, and the *Sal*I–*Not*I fragment was cloned into corresponding sites of pCMV-Myc (Clontech). This fragment encodes a slightly shorter polypeptide than the full-length protein, encompassing amino acids 1–1146. For construction of the full-length protein, extension of the original Nek1–not12 fragment was performed using a 3′ fragment from the not18 clone [23]. Site-directed mutagenesis at the ATP-binding site (G13R) was carried out using the QuikChange kit (Stratagene, La Jolla, CA), with primers: 5′ CAGAAGATTGGAGAA***C***GTTCATTTGGAAAAG 3′ and 5′ CTTTTCCAAATGAAC***G***TTCTCCAATCTTCTG 3′. Wild type and G13R full-length vectors were then truncated by cutting with *Bgl*II and *Not*I, followed by mung bean nuclease digestion and self-ligation (resulting in constructs N-1001, and N-1001^{G13R}). An additional truncation, using *Xcm*I and *Not*I resulted in construct N-753. Removal of an internal *Xho*I fragment resulted in the $FL^{\Delta 151-438}$ construct. The first NLS mutations (K365 R366/AA) were generated using the QuikChange kit and the primers: CTGAGGAAGCAGCAAAA***GC***A***G***-AAGGTTGGAATTTATTGAGAAAG and CTTTCTCAATAAATTCCAA-CCTT***GC***T***GC***TTTTGCTGCTTCCTCAG, and the second NLS mutation K581R582/AL) using the primers: GAAGACCTGGACCGA***GCTCTG***AAG-***CAGAGC***TCGCTCCAGCTGTTC. NES mutations (L1131G, L1134A) were generated using the primers: 5′ CAGTGTCTTTAACCAT***GG***AGAGGAA***G***-AAGACTTCACTTGGAGCAAG 3′ and 5′ CTTGCTCCAAGTGAAGTC-TT***GC***TTCCTCT***CC***ATGGTTAAAGACACTG 3′ using the QuikChange kit. The 764 NES sequence was mutated (L644G, L767S) using the primer ATGGCTCTCCAAGAAAAGTCTGGGGGAAAAACCCTACAGATTCTG-TG***GG***GAAGATA***TC***TGGAGAAGCTGAATTACAGC in a PCR reaction.

2.2. Sequence analyses

PEST predictions were run online at http://www.at.embnet.org/embnet/tools/bio/PESTfind/, at a window of 10.

Nuclear localization signal (NLS) predictions were performed at http://cubic.bioc.columbia.edu/predictNLS/.

2.3. Transfections

HeLa, NIH3T3 and HEK293 cells were transiently transfected using CellPhect Transfection kit (Amersham, Pharmacia Biotech) according to the manufacturer's instructions.

2.4. Immunocytochemistry

Polyclonal antibodies against Nek1 were raised against two different domains: I. A *Sph*I–*Hin*dIII fragment encoding the N-terminal 187 amino acids (and 2 amino acids upstream of the initiating methionine), inserted into the pQE30 vector (Qiagen,Valencia, CA) downstream and in-frame with a 6XHis tag. II. A *Bgl*II–*Not*I fragment encoding the C-terminal 204 amino acids inserted into the pGEX-4T-2 vector downstream and in-frame with the GST tag. Purified proteins were injected into rabbits, and the resulting antibodies were affinity-purified using the corresponding Nek1 fragment coupled to an activated cyanogen bromide Sepharose column (Sigma). Antiserum was passed over the column and eluted with 100 mM glycine, pH 2.5, into 1 M Tris, pH 9.5.

For immunofluorescence microscopy, cultures grown on coverslips were fixed in 4% paraformaldehyde for 20 min., blocked with 3% BSA/0.5% Triton X-100 in PBS, and incubated overnight with primary antibodies at 4 °C. Primary antibodies were used as follows: Nek1 purified polyclonal antibodies diluted 1:50, Myc-monoclonal antibodies (Zymed Laboratories, CA) 1:250, HA-monoclonal antibodies (BAbCO, Richmond, CA) 1:1000, mouse α/β Tubulin (Biomeda, Foster City, CA) 1:1000, rabbit anti P-S10-Histone-H3 (Upstate Biotechnology, NY) 1:100, and rabbit anti activated Caspase-3 (Cell Signaling) diluted 1:75. Following washes, the coverslip was incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse and/or Rhodamin-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) (1:150). DNA staining was done with propidium-iodide solution, and the coverslips were mounted on slides with Vectashield mounting solution (Vector, Burlingame, CA) and sealed. Staining was analyzed using an MRC 1024 confocal laser microscope (Bio-Rad).

2.5. Cell fractionation

Cells were washed twice by PBS and harvested by lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris–HCl pH=7.5, 0.5% NP-40). The extracts were homogenized by a syringe needle (21G, 25 times), and centrifuged for 3 s at 20,000×g at room temperature. SDS sample buffer was added to the supernatant that were marked as cytoplasm. The pellet was washed twice by lysis buffer, followed by spin down for 5 s. The pellet was dissolved by SDS sample buffer and marked as nucleus.

3. Results

3.1. Nek1 protein structure is highly similar to that of NIMA

The murine Nek1 kinase was reported to consist of 774 amino acids, with a calculated molecular weight of 88.4 kDa, and limited structural homology to NIMA [23]. However, antibodies raised against the N-terminus portion of Nek1 detected a prominent band at about 180 kDa in Western analysis of various murine tissue extracts, indicating that the translated product is larger than originally predicted (see below). We thus compared the reported *nek1* cDNA sequence to the deposited murine sequences in the various data bases (genomic, cDNAs and ESTs), and sequenced one of the original clones (clone 23, described in [23]). The sequence analysis suggested that two reading mistakes occurred in the original report (T instead of C was reported at nucleotide 2898, and G following nucleotide 3869 was omitted; the corrected sequence has been deposited in NCBI GenBank Accession No. AY850065). According to the corrected open reading frame, the murine Nek1 encodes a 1203 amino acid protein, with a calculated molecular weight of 136.7 kDa (Fig. 1A). Nek1 is the longest documented NIMA-family kinase, bearing a 945 C-terminal non-catalytic tail. As the C-terminus is highly enriched in acidic residues, the overall