DdNek2, the first non-vertebrate homologue of human Nek2, is involved in the formation of microtubule-organizing centers

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Summary

Dictyostelium **Nek2 (DdNek2) is the first structural and functional non-vertebrate homologue of human Nek2, a NIMA-related serine/threonine kinase required for centrosome splitting in early mitosis. DdNek2 shares 43% overall amino-acid identity with its human counterpart and 54% identity within the catalytic domain. Both proteins can be subdivided in an N-terminal catalytic domain, a leucine zipper and a C-terminal domain. Kinase assays with bacterially expressed DdNek2 and C-terminal deletion mutants revealed that catalytic activity requires the presence of the leucine zipper and that autophosphorylation occurs at the C-terminus. Microscopic analyses with DdNek2 antibodies and expression of a GFP-DdNek2 fusion protein in** ***Dictyostelium*** **showed that DdNek2 is a permanent centrosomal resident and suggested that it is a component of the centrosomal core. The GFP-DdNek2-overexpressing mutants frequently exhibit supernumerary microtubule-organizing centers (MTOCs). This phenotype did not require catalytic activity because it was also observed in cells expressing inactive GFP-K33R. However, it was shown to be caused by overexpression of the C-terminal domain since it also occurred in GFP-mutants expressing only the C-terminus or a leucine zipper/C-terminus construct but not in those mutants expressing only the catalytic domain or a catalytic domain/leucine zipper construct. These results suggest that DdNek2 is involved in the formation of MTOCs. Furthermore, the localization of the GFP-fusion proteins revealed two independent centrosomal targeting domains of DdNek2, one within the catalytic or leucine zipper domain and one in the C-terminal domain.**

Key words: *Dictyostelium*, Centrosome, Spindle pole body, NIMA-related kinase, Nek2

Introduction

NIMA-related kinases are a ubiquitous family of serine/threonine kinases that promote cell-cycle-dependent events. The first member of this kinase family, NIMA, was identified in *Aspergillus nidulans* where temperature-sensitive *nimA* mutants arrested in G2 and, thus, failed to enter mitosis under restrictive conditions (hence the name nimA – 'never in mitosis') (Osmani et al., 1991). Subsequent studies revealed that NIMA possesses several regulatory elements for rapid degradation and phosphorylation by Cdk1/-cylin B (O'Connell et al., 1994; Pu et al., 1995; Ye et al., 1995) and is needed for nuclear localization of Cdk1-cylin B (Wu et al., 1998). Overexpression of the wild-type gene causes premature mitosis and induces chromosome condensation (Osmani et al., 1988). Among other organisms, *Neurospora crassa* NIM-1 is so far the only real homologue of NIMA that shows not only high sequence similarity but also fulfills the same cellular function.

However, serine/threonine kinases related to NIMA, but with other cellular functions, have been identified in many organisms. In humans, at least six NIMA-related kinases have so far been isolated as full-length or partial sequences (Levedakou et al., 1994; Lu and Hunter, 1995; Schultz et al., 1994; Schultz and Nigg, 1993). Among these, Nek2 is most closely related to NIMA. But instead of promoting mitosis, it has a centrosomal function in centrosome separation (Fry et al., 1998b). In animal cells, the first step of centrosome duplication (Kochanski and Borisy, 1990), that is, the formation of daughter centrioles, is initiated at the G1/S transition. In early prophase, the centrosome consists of two complete centriole pairs and their pericentriolar matrix, defining two centrosomal entities. The centriole pairs are linked by a fibrous structure (Paintrand et al., 1992) that is associated with a long coiled coil protein called C-Nap1 (Fry et al., 1998a; Mayor et al., 2000). C-Nap1 (which had been independently characterized as Cep250) (Mack et al., 1998) was identified in a yeast two-hybrid screen using human Nek2 as a bait (Fry et al., 1998a). Nek2 plays a key role in centriole/centrosome separation in early mitosis by phosphorylation of C-Nap1. This seems to trigger the dissociation of C-Nap1 from the centrosome, which in turn leads to a loss of centriole cohesion, allowing the two centrosomal entities to separate and organize the mitotic spindle (Fry et al., 1998a; Mayor et al., 2000). Protein phosphatase 1α (PP1α), another binding partner of Nek2, acts as a physiological antagonist of Nek2 and seems to stabilize centrosome cohesion by dephosphorylation of Nek2 and C-Nap1 (Helps et al., 2000; Meraldi and Nigg, 2001). Analysis of Nek2B in *Xenopus* embryos revealed an additional function of Nek2 in centrosome maintenance and assembly. Nek2B

inhibition by antibody injection or overexpression of catalytically inactive, dominant-negative Nek2B caused centrosome dispersal (Uto and Sagata, 2000), and Nek2B immunodepletion delayed the assembly of components of the pericentriolar matrix, including γ-tubulin and Nek2B, at the sperm basal bodies in egg extracts (Fry et al., 2000a).

Dictyostelium discoideum amoebae have become an interesting model system for the comparative analysis of centrosomes (Daunderer et al., 1999) because of their good structural characterization (Moens, 1976; Roos, 1975), the availability of a centrosome isolation protocol (Gräf et al., 1998), the genome project (Kay and Williams, 1999) and the molecular characterization of centrosomal components, such as γ-tubulin (Euteneuer et al., 1998), DdCP224 (Gräf et al., 2000b) and the homologues of centrin (DdCrp) (Daunderer et al., 2001), Spc97 and Spc98 (Gräf et al., 2000a). The *Dictyostelium* centrosome represents an acentriolar centrosome type with a unique mode of duplication (Ueda et al., 1999). In interphase, the *Dictyostelium* centrosome consists of a box-shaped, layered core structure surrounded by an amorphous matrix called the corona. The corona harbors electron-dense nodules containing γ-tubulin and, thus, the microtubule-nucleation sites (Euteneuer et al., 1998). Centrosome duplication starts in early prophase with an enlargement of the layered core and dissociation of the surrounding corona with its emanating microtubules. In prometaphase, the central layer disappears and the two outer layers peel apart and become inserted into the nuclear envelope. Microtubules are nucleated from the inner surfaces of the two layers and form an elongating spindle, which separates the two spindle poles. During the course of the separation process, the edges of the plaque-like poles bend away from the nucleus and each plaque folds back onto itself in telophase. The microtubule-nucleating surface is now exposed to the outside, whereas the former outside becomes buried inside. The new inner layer matures in late telophase. Thus, similar to mammals but unlike yeast where the new spindle pole body consists only of freshly assembled components (Adams and Kilmartin, 2000), the *Dictyostelium* centrosome duplicates in a semiconservative manner, as each daughter centrosome originates from one of the two former outer layers (Gräf et al., 2000a). With regard to the time point of centrosome duplication, *Dictyostelium* is unique because duplication starts late in the cell cycle, in M-phase, and is not synchronized with the G1/S transition as in budding yeast or animals. This raises the question of whether this process can be regulated by a similar set of kinases (for a review, see Fry et al., 2000b).

To address this issue in *Dictyostelium*, this study describes the characterization of a NIMA-related kinase, which represents the first non-vertebrate homologue of Nek2. This kinase, named DdNek2, is structurally related to Nek2, is an integral centrosomal component and plays a role in the formation of MTOCs.

Materials and Methods

Cell culture

Vegetative *Dictyostelium* cells were grown as described previously (Gräf et al., 1999). Green fluorescent protein (GFP)-DdNek2 mutants were grown at 21°C in HL-5c medium containing 4 μg/ml blasticidin S (ICN Biomedicals, Eschwege, Germany) or 5 μg/ml G418 (Sigma, Deisenhofen, Germany), respectively.

Protein expression and generation of polyclonal antibodies

Clone SLD805 from the Tsukuba cDNA project (kindly provided by Y. Tanaka) was sequenced completely (MWG-Biotech, Ebersberg, Germany; the sequence can be retrieved at http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html). The complete coding sequence (position 53-1306) was cloned into the bacterial expression vector pMAL-c2 (NEB, Schwalbach, Germany) as described recently (Gräf, 2001b). DdNek2, C-terminally fused to maltose-binding protein (MBP), was expressed in *E. coli* XL-1 blue cells and purified on an amylose resin (NEB). The purified fusion protein was used for the immunization of two rabbits (J. Pineda, Antikörperservice, Berlin, Germany). Antisera were taken on the 61st day, following five immunizations with ~0.2 mg antigen each. Antibodies were purified by affinity chromatography on columns with immobilized MBP-DdNek2. The fusion protein was coupled to NHS-activated Sepharose (NHS Sepharose 4B, Pharmacia). Specific antibodies were eluted with 100 mM glycine, pH 2.7 and neutralized immediately by addition of a droplet of 1 M Tris/Cl, pH 8.7.

The C-terminal deletion mutants MBP-Δ315-N, MBP-Δ267-N and MBP-Δ258-C were prepared as follows. The respective coding sequence was amplified by the polymerase chain reaction (PCR) using an upstream *Eco*RI-linker primer binding at base position 53-75 and downstream *Xba*I-linker primers binding at base position 830-853 (MBP-Δ267-N) and 977-997 (MBP-Δ315-N), respectively. The binding sites for *Eco*RI/*Xba*I linker primers for construction of the N-terminal deletion construct MBP-Δ258-C were 830-854 (upstream) and 1287-1309 (downstream). Clone SLD805 was used as the template for PCR. The DNA fragments were cloned into pMAL-c2 using the *Eco*RI and *Xba*I restriction sites, and proteins were expressed and purified as described above. If MBP-fusion proteins were used for kinase activity assays, a column buffer containing 50 mM Hepes/K (pH 7.4), 150 mM KCl, 2 mM $MgCl_2$ and 1 mM dithiotreitol (DTT) was used, and fusion proteins were eluted with kinase buffer (50 mM Hepes/K (pH 7.4), 5 mM $MnCl_2$, 5 mM β-glycerophosphate, 1 mM DTT) containing 10 mM maltose.

Construction of GFP-expression vectors

C-terminal fusions of DdNek2 with GFP showed only a weak fluorescence and were not detectable at the expected cellular localization. The phenotypes of N-terminal GFP-fusion mutants were not affected by the vector system used. The first vector used was pDGFPMCS (Weber et al., 1999), where expression is driven by the actin15 promoter. The second was the Tet-off system, which was based on MB38/MB35 (Blaauw et al., 2000). MB38 was cut with *Bgl*II and *Mlu*I and modified by insertion of superglow-GFP (Qbiogene, Heidelberg, Germany) followed by a *Sal*I-*Sac*I-*Eco*RI-*Bam*HI polylinker. The third, a 7.75 kb plasmid named pDiscGFPSSEB2, drives expression of the protein of interest by the discoidin promoter (Wetterauer et al., 1995). The promoter is followed by superglow-GFP, the *Sal*I-*Sac*I-*Eco*RI-*Bam*HI linker, the actin 8 terminator, the V18-Tn5 selection cassette for G418 selection (Wetterauer et al., 1996) on *Klebsiella aerogenes* lawns and an ampicillin resistance cassette. All DdNek2 full-length and deletion constructs were inserted in these vectors employing the *Sal*I and *Eco*RI restriction sites, and all DdNek2 fragments were generated by PCR using *Sal*I- and *Eco*RI-linker primers and the original SLD805 clone as a template. The base positions for primer-binding sites on SLD805 for upstream and downstream primers, respectively, were 53-75/1287-1309 for GFP-DdNek2 and GFP-K33R, 53-75/830-853 for GFP-Δ267-N, 53-75/977-997 for GFP-Δ315-N, 830-854/1287-1309 for GFP-Δ258-C and 986-1007 for GFP-Δ312-C. Sequences were confirmed by custom sequencing (Delphiseq, Regensburg, Germany).

A

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Dd      MDQYEILGALGKGSFGVVSKIKRKEDGRVLVWKEICYENMQEKEKQLLVNEVNILQKLKHQNIVRYYDRIIDKPSSRLYIIMEHCSGGDLSQLIKK 96
Hs MPSRAEDYEVLYTIGTGSYGRCQKIRRKSDGKILVWKELDYGSMTEAEKQMLVSEVNLLRELKHPNIVRYYDRIIDRTNTTLYIVMEYCEGGDLASVITK 100

Dd CRNERTYMDEEVIWRTLLQILSALQEIHNRKDG..VILHRDIKPGNLFLDENKNIKLGDFGLAKILN.ESLYAHTFVGTPYYMSPEQIHGLKYNERSDVW 193
Hs GTKERQYLDEEFVLRVMTQLTLALKECHRRSDGGHTVLHRDLKPANVFLDGKQNVKLGDFGLARILNHDTSFAKTFVGTPYYMSPEQMNRMSYNEKSDIW 200

Dd SVGCLIYEMATLSPPFEATNQAQLTSKIQVGRYNPIPSQYSEHLSKVISLMINVDPKSRPNVNELLGYSFI/SFKV.KERKLNIYYQGL......KQMD. 283
Hs SLGCLLYELCALMPPFTAFSQKELAGKIREGKFRRIPYRYSDELNEIITRMLNLKDYHRPSVEEILENPLI/ADLVADEQRRNLERRGRQLGEPEKSQDS 299

Dd ....EDLKIKEKKLSDIERDLQVKEQHLLLREQQINQREKLLLDKENFETQSRINIMNQQLQQQQQ...NQLQHQISNLSLNCNNSVNSCSSSSNNNTTN 377
Hs SPVLSELKLKEIQLQERERALKAREERLEQKEQELCVRERLAEDK...LARAENLLKNYSLLKERKFLSLASNPELLNLPSSVIKKKVHFSGESKENIMR 396

Dd SINTQQQIHIQHNTQQQQQQQQTFQPYQIKRTFTTPL.PNFK..... 418
Hs SENSESQLTSKSKCKDLKKR...LHAAQLRAQALSDIEKNYQLKSRQILGMR 445
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B

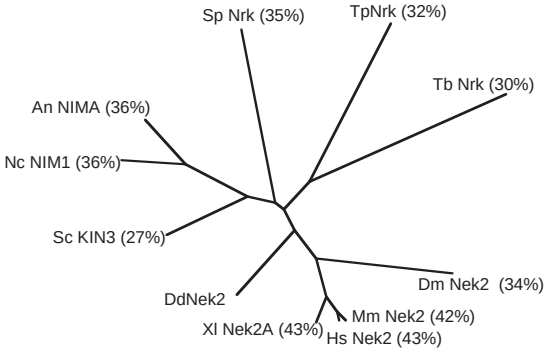

Fig. 1. Phylogenetic relationship between DdNek2 and other NIMA-related kinases. (A) Pairwise alignment of the DdNek2 and human Nek2 amino-acid sequences using the ClustalW program. Conserved residues specific for NIMA-related kinases are highlighted by the black boxes and residues common to all serine/threonine kinases appear in light gray boxes. The lysine residue that was replaced by arginine in the catalytically inactive kinases is printed in bold and boxed in gray. The six heptads of the leucine zipper are underlined and the end of the catalytic domain is marked by a slash. (B) Phylogenetic trees derived from multiple alignments of all relevant NIMA-related kinases using the complete DdNek2 coding sequence. The alignments were made with the ClustalW program and the trees calculated with the PHYLIP package, version 3.5c. Numbers in brackets refer to the percentage of amino-acid identity to DdNek2. Abbreviations and accession numbers are: *Dictyostelium discoideum* DdNek2 (SLD805), *Xenopus laevis* Xl-Nek2A (Q9W622), *Mus musculus* Mm-Nek2 (O35942), *Homo sapiens* Hs-Nek2 (P51955), *Drosophila melanogaster* Dm-Nek2 (Q9W3N8), *Schizosaccharomyces pombe* Sp-Nrk (O13839), *Saccharomyces cerevisiae* Sc-KIN3 (P22209), *Neurospora crassa* Nc-NIM1 (P48479), *Aspergillus nidulans* An-NIMA (P11837), *Tetrahymena pyriformis* TpNrk (O76134) and *Trypanosoma brucei* Tb-Nrk (Q08942).

Generation of the DdNek2-K33R point mutant

The DdNek2 K33R point mutation, where the A at base position 150 was replaced with G, was generated by PCR in a two-step approach. Two fragments were amplified, the first, using the mutagenesis primer GATGGAAGAGTTTTAGTTTGGA**G**AGAAATTTGTTATG (base positions 128-164) and a downstream *Nsi*I-linker primer (binding at base position 1287-1309), and the second, using the complementary mutagenesis primer and an *Bam*HI upstream linker primer (binding at base position 53-75). The PCR products were purified with QiaQuick columns (Qiagen, Hilden, Germany) and used as a template for the second PCR reaction with the *Bam*HI upstream and *Nsi*I downstream primers only. Since the single-stranded fragments from the first reaction are complementary to each other in the mutagenesis primer sequence they will anneal in the first annealing step, and the complete coding sequence including the point mutation will be obtained in the first extension step. This is the only possible template for the *Sal*I upstream and *Eco*RI downstream primers, since the mutagenesis primers have been removed in the purification step. The resulting PCR product was cloned into p1ABsr8 (Gräf et al., 2000b) using the *Bam*HI and *Nsi*I restriction sites. The complete DdNek2-K33R sequence was then amplified using the *Eco*RI upstream and *Xba*I downstream primer for cloning into pMAL-c2 or the *Sal*I upstream and *Eco*RI downstream primer (see above) for cloning into the GFP-vectors. After these cloning steps, sequences were confirmed by custom sequencing (Delphiseq, Regensburg, Germany).

Transformation into *Dictyostelium* cells

Plasmids were transformed into AX2 cells using electroporation (Mann et al., 1998), and clones were selected with 4 μg/ml blasticidin S in liquid culture in the case of pDGFPMCS- and MB38-based constructs and with 100 μg/ml G418 on *Klebsiella aerogenes* plates (Wetterauer et al., 1996) in case of pDiscSSEB2-based constructs. In the latter case, mutant clones were further grown in liquid medium containing 10 μg/ml G418 after the selection step. At least three independent clones were analyzed for each transformant and, except for minor differences in GFP fluorescence intensity, no differences between corresponding clones were observed.

Kinase activity assays

In vitro kinase activity of the MBP-fusion proteins was assayed according to Fry and Nigg (Fry and Nigg, 1997) as described recently (Gräf, 2001b). In brief, 100 μl reactions in kinase buffer contained 5 μg of dephosphorylated α/β-casein (Sigma, Deisenhofen, Germany) or BSA as a negative control, 5 μg of the purified fusion protein, 2 μg/ml heparin, 4 μM ATP and 10 μCi of γ-[^{32}P]-ATP (3000 Ci/mmol; ICN Biomedicals, Eschwege, Germany). After incubation for 1 hour at 30°C, proteins were concentrated by trichloroacetic acid precipitation (Bollag et al., 1996) and subjected to SDS gel electrophoresis. The dried gel was exposed overnight on X-ray film (Kodak X-OMAT AR5) at –70°C.

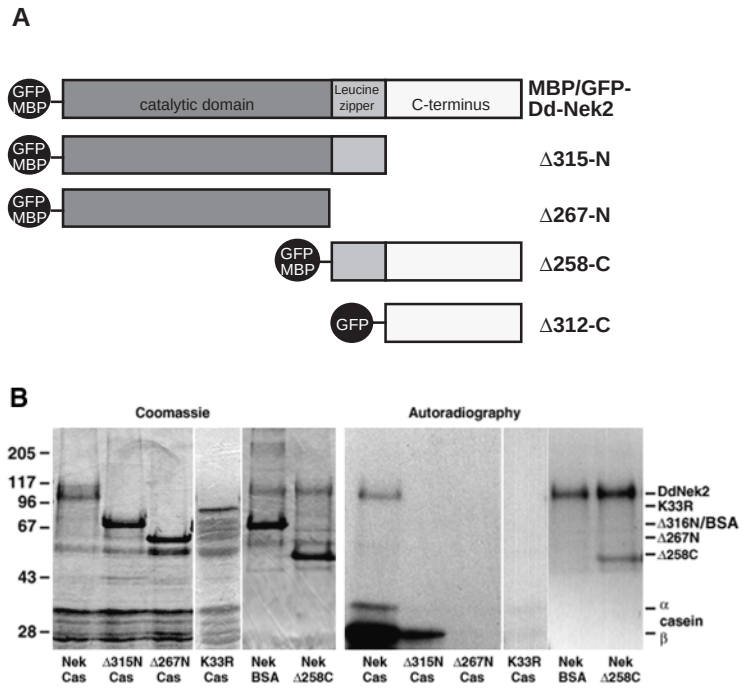

Fig. 2. (A) An overview of DdNek2 deletion mutants. (B) Catalytic activity of MBP-DdNek2 and its mutants. The substrates were α/β-casein, the recombinant C-terminal DdNek2 fragment MBP-Δ258-C and BSA (negative control). DdNek2 and its C-terminal deletion constructs were analyzed on the same gel to allow comparison of their catalytic activity. Proteins of each kinase reaction precipitated with TCA were separated by SDS-gel electrophoresis, stained with Coomassie and subjected to autoradiography. The molecular mass (kDa) of standard proteins is indicated on the left and the position of the individual protein bands on the right. The kinase derivatives and substrates used are given at the bottom. *Nek* refers to DdNek2, *K33R* to MBP-DdNek2-K33R, *Cas* to α/β-casein, *Δ258C* to MBP-Δ258-C, *Δ315N* to MBP-Δ315-N and *Δ267N* to MBP-Δ267-N.

Image processing

Confocal microscopic image stacks were processed with the Huygens 2.2 deconvolution software (Bitplane AG, Zürich, Switzerland) using a computed theoretical point spread function and the Maximum Likelihood Estimation algorithm.

Other methods

SDS polyacrylamide electrophoresis, immunoblotting, light microscopy, indirect immunofluorescence labeling, centrosome isolation and preparation of cytosolic protein extracts were performed as described recently (Daunderer et al., 2001; Gräf, 2001a).

Results

DdNek2 is most closely related to the mammalian and *Xenopus* Nek2 proteins

DdNek2 was identified on clone SLD805 in the data library of the Tsukuba cDNA project (Morio et al., 1998) by its high homology to human Nek2. SLD805 was sequenced completely and has a length of 1373 bp (the sequence can be retrieved at http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html). The cDNA includes the complete coding sequence for a 48.9 kDa protein with a similar size to human Nek2 (Schultz et al., 1994) to which it also shows its highest homology (Fig. 1A). The two proteins share 43% identity in their amino acids over the full-length sequence and even 54% within the catalytic domain. Only mouse Nek2 (Rhee and Wolgemuth, 1997; Tanaka et al., 1997) and *Xenopus* Nek2A (Fry et al., 2000a; Uto et al., 1999) show a similar degree of sequence conservation whereas the other members of the NIMA-related kinase family are clearly less closely related to DdNek2 (Fig. 1B). The only other non-vertebrate Nek2 homologue, the *Drosophila* protein, is almost twice as large as the *Dictyostelium*, human, mouse and *Xenopus* sequence. The deduced DdNek2 amino-acid sequence not only contains all relevant characteristics of serine/threonine kinases and the diagnostic sequence motifs LY(I/L)XM(E/D)YCXG-GDL and CX(L/M)YECC of NIMA-related kinases (Schultz et al., 1994), it also shares a similar domain structure with human Nek2 (Fig. 1A). The N-terminal catalytic domain is followed by a leucine zipper and a regulatory C-terminal domain. The leucine zipper motif lies within a coiled coil domain (amino-acid position 270-346; confirmed using the coilscan program (Lupas et al., 1991) with a 28 amino-acid window size) and is longer than the usual four to five heptads (see Discussion).

Catalytic activity of recombinant DdNek2 requires the leucine zipper domain

The catalytic properties of DdNek2 and several deletion constructs were tested by in vitro kinase assays. This was facilitated by the fact that DdNek2 expressed as a MBP-fusion protein in *E. coli* was catalytically active, unlike its human homologue, which could not be functionally expressed in bacteria using the same system (Fry and Nigg, 1997). In these assays, DdNek2 exhibited the typical properties of Nek2 kinases, that is, it underwent autophosphorylation and was able to phosphorylate the artificial substrates casein (Fig. 2), histone H1 and myelin basic protein (not shown). As expected, activity was stronger with β-casein than with α-casein. The catalytically inactive DdNek2-K33R point mutant (MBP-K33R) corresponding to the human K37R mutant (Fry et al., 1995), where a critical lysine in the catalytic domain was replaced by an arginine residue, showed a higher electrophoretic mobility owing to the lack of phosphate groups (Fig. 2B). The requirement of the leucine zipper and C-terminal domain for kinase activity was investigated with DdNek2 deletion mutants (Fig. 2A). The catalytic domain alone (MBP-Δ267-N) was inactive, whereas the mutant consisting of the catalytic and dimerization domains (MBP-Δ315-N) retained a weak β-casein kinase activity but no autophosphorylation activity. This suggests that the leucine zipper is essential for catalytic activity and that autophosphorylation increases kinase activity, as in human Nek2, where the leucine zipper is required for dimerization and each catalytic domain within the Nek2 dimer trans-autophosphorylates the C-terminus (Fry et al., 1999). Since the MBP-Δ315-N mutant, where the C-terminal domain was deleted, was not autophosphorylated, this domain is likely to contain the autophosphorylation target site, similar to human Nek2. This hypothesis was supported by experiments where a

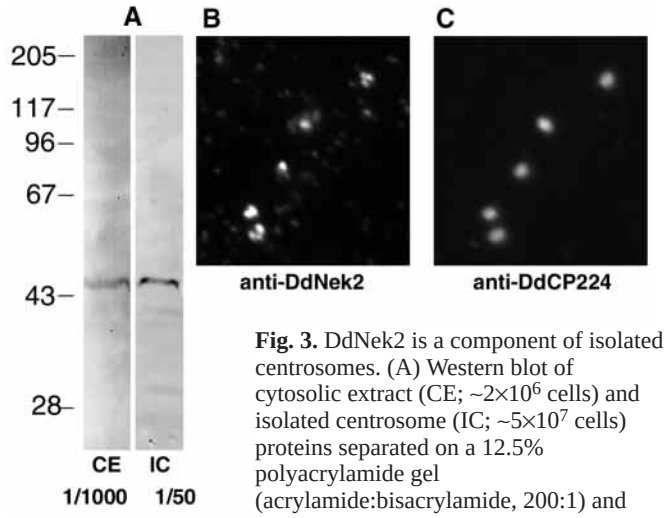

Fig. 3. DdNek2 is a component of isolated centrosomes. (A) Western blot of cytosolic extract (CE; ~2×10^6 cells) and isolated centrosome (IC; ~5×10^7 cells) proteins separated on a 12.5% polyacrylamide gel (acrylamide:bisacrylamide, 200:1) and stained with anti-DdNek2 antibodies. (B,C), Immunofluorescence microscopy of isolated centrosomes spun down to coverslips, fixed with methanol and stained with rabbit anti-DdNek2 (B) and mouse anti-DdCP224 antibodies (C). Secondary antibodies were Alexa-568-labeled anti-rabbit IgG and Alexa-488-labeled anti-mouse IgG. Bar, 2 μm.

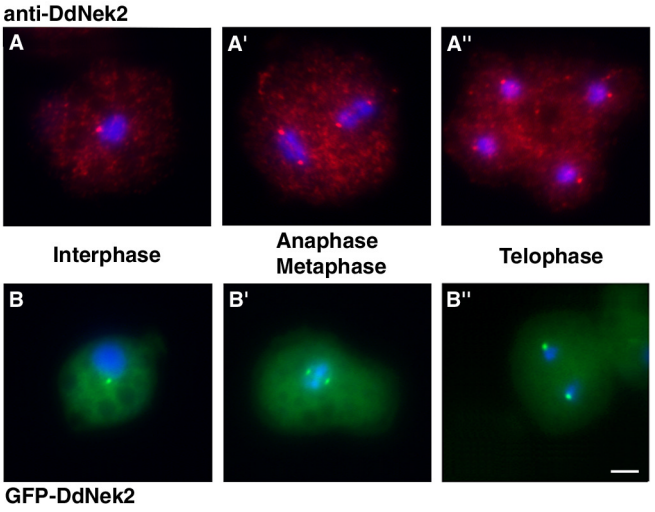

Fig. 4. DdNek2 resides at the centrosome throughout the entire cell cycle. Fluorescence microscopy of *Dictyostelium* AX2 cells labeled with anti-DdCP224 antibodies (A-A″) and GFP-DdNek2 cells (B-B″). Cells were fixed with methanol. The secondary antibody in (A-A″) was Cy3-labeled anti-mouse IgG. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Bar, 2 μm.

deletion mutant lacking the catalytical domain (MBP-Δ258-C) was used as a substrate for MBP-DdNek2 in the kinase activity assay. Phosphorylation of MBP-Δ258-C was relatively weak compared with autophosphorylation of MBP-DdNek2. This is not surprising, since the mechanism of trans-autophosphorylation within the kinase dimer does not require binding of a substrate, whereas MBP-Δ258-C needs to bind to the active dimer before it can be phosphorylated. The affinity of MBP-DdNek2 for MBP-Δ258-C is likely to be rather low since, in vivo, the mechanism of trans-autophosphorylation requires no binding site for the C-terminal domain within the complete, dimerized kinase.

DdNek2 is a genuine centrosomal component and localized to the centrosome throughout the entire cell cycle

Two rabbits (rabbit 1 and 2) were immunized with recombinant MBP-DdNek2. Both antisera showed similar staining patterns in western blots and immunofluorescence microscopy; however, the rabbit 1 serum turned out to be more specific and was used in all experiments presented here. Preimmune antibodies and anti-MBP antibodies showed no crossreactions or background staining when used at a comparable concentration (data not shown). On western blots of cytosolic extracts and isolated centrosome preparations, anti-DdNek2 antibodies stained a single band of ~46 kDa, which was close to the calculated molecular mass of DdNek2 (Fig. 3A). Furthermore, the antibodies clearly stained isolated centrosomes in immunofluorescence microscopy (Fig. 3B,C). Since isolated *Dictyostelium* centrosomes are devoid of microtubules (Gräf et al., 1998), centrosomal localization of DdNek2 is independent of microtubules. Furthermore, anti-Dd-Nek2 stained the centrosome throughout the entire cell cycle in immunofluorescence microscopy of vegetative AX2 cells (Fig. 4A). This localization pattern was confirmed when DdNek2 was expressed as a GFP-fusion protein in AX2 cells (Fig. 4B). Taken together, these results demonstrate that DdNek2 is a genuine centrosomal component. The existence of a large cytosolic DdNek2 pool does not contradict this finding since it is also the case for human Nek2 and common for many, if not most, centrosomal proteins, including classical centrosomal components such as γ-tubulin (Moudjou et al., 1996) and centrin (Paoletti et al., 1996). However, it may argue for further cytosolic functions of this kinase as well.

GFP-DdNek2 overexpression causes formation of supernumerary MTOCs

Many of the cells expressing GFP-DdNek2 were only weakly fluorescent and rarely showed a mutant phenotype other than faintly green-fluorescent centrosomes. However, the majority of cells exhibiting a high level of GFP-DdNek2 expression in fluorescence microscopy (~20% of all cells) displayed aberrations in centrosome number and morphology, nuclear size and shape, or contained multiple GFP foci (Fig. 5A-E; Table 1). These defects also occurred in combination. The segregation into a weakly and a strongly fluorescent cell population was independent of the promoter used for expression (see Materials and Methods). Most likely, overexpression of the kinase affects cell division and, thus, there is a permanent selective pressure in favor of cells with an attenuated GFP-DdNek2 expression. Among the overexpressing cells only ~30% are more or less normal, that is, they had only one green, nucleus-associated centrosome. In contrast, if cells were transformed with a GFP-vector without an insert, almost 95% of all cells were normal (Table 1). About 25% of all GFP-DdNek2 cells contained GFP-fluorescent dots, often more than 10, which did not stain with γ-tubulin antibodies (Fig. 5E) but which might include other centrosomal proteins in addition to GFP-DdNek2. Misshapen

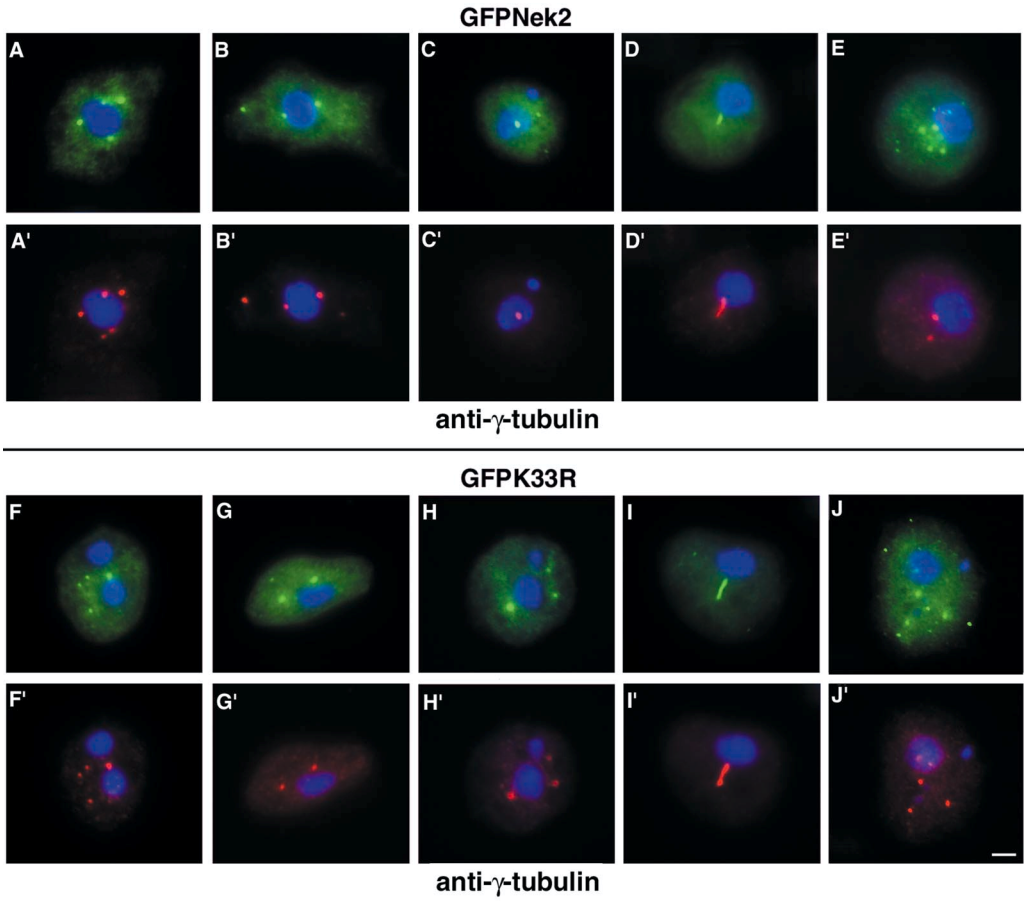

Fig. 5. Overview of the phenotypes of GFP-DdNek2 (A-E) and GFP-K33R (F-J) mutants. Immunofluorescence microscopy of cells fixed with methanol and labeled with anti-γ-tubulin (A′-J′). The secondary antibody was Alexa-568-labeled anti-rabbit IgG. Nuclei were stained with DAPI. The main phenotypes shown are supernumerary MTOCs (A,B,F,G,H,E,J), nuclear aberrations (C,H,J), misshapen centrosomes (D,I) and multiple GFP foci (E,J). Bar, 2 μm.

centrosomes that do not occur in wild-type cells were also observed (Fig. 5D). In some cases, cells also contained unusually large nuclei or additional, very small nuclei (Fig. 5C), both indicating defects in proper chromosome segregation. About 40% of the strongly fluorescent cells contained supernumerary MTOCs that could be stained with anti-γ-tubulin antibodies. The presence of the four centrosomal markers γ-tubulin (Euteneuer et al., 1998), DdCP224 (Gräf et al., 2000b), DdNek2 and the NAB350 antigen (Kalt and Schliwa, 1996) as well as the capacity to nucleate microtubules suggest that these MTOCs represent complete centrosomes (Figs 5 and 6). Furthermore, confocal microscopy revealed that both extra MTOCs and normal, nucleus-associated centrosomes appear as doughnut-like structures of approximately the same size when stained with anti-DdCP224 antibodies (Fig. 6A′,D′-F′). This staining pattern appears because DdCP224 is part of the centrosomal corona that surrounds the centrosomal core (Gräf et al., 2000b). In merged confocal images of DdCP224 and DdNek2 localization (Fig. 6D″) and in tracings of fluorescence intensity along a line through the center of the centrosomes (Fig. 6D‴), it becomes evident that the DdNek2-labeled part of the centrosome is surrounded by the corona. This suggests that DdNek2 is associated with the centrosomal core structure. This staining pattern is indistinguishable in normal centrosomes and supernumerary MTOCs. Taken together, overexpression of GFP-DdNek2 causes the formation of centrosome-like, supernumerary MTOCs.

The defects of GFP-DdNek2 cells are not dependent on catalytic activity but are linked to overexpression of the C-terminal domain

It was tempting to speculate that the observed abnormalities were caused by an increase of DdNek2 activity owing to overexpression. Therefore, catalytically inactive DdNek2-K33R was also expressed in *Dictyostelium* as a GFP-fusion protein. Surprisingly, the phenotypes of the GFP-K33R mutants were indistinguishable from the GFP-DdNek2 mutants in all their aspects, including the occurrence of supernumerary MTOCs, misshapen centrosomes, multiple GFP-foci and nuclear abnormalities (Fig. 5; Table 1). Again, there was no dependence on the promoters used. Thus, the mutant phenotype cannot be caused by an overdose of catalytic activity. To elucidate the cause of the mutant phenotype, GFP fusions with DdNek2 deletion mutants were created (Fig. 3). *Dictyostelium* mutants expressing only the catalytic domain with GFP (GFP-Δ267-N) showed no centrosomal labeling but they had high overall cytosolic fluorescence (Fig. 7A,B). If the leucine zipper sequence was present as well, the resulting fusion protein (GFP-Δ315-N) clearly localized to the centrosome (Fig. 7C,D). Supernumerary MTOCs or other striking abnormalities were detected neither in GFP-Δ267-N nor in GFP-Δ315-N mutants (Fig. 7A-D). However, two further deletion mutants, GFP-Δ258-C and GFP-Δ312-C, which expressed the C-terminus/leucine zipper construct and the C-terminus alone, respectively, localized to centrosomes and exhibited the same mutant phenotypes as GFP-DdNek2 and

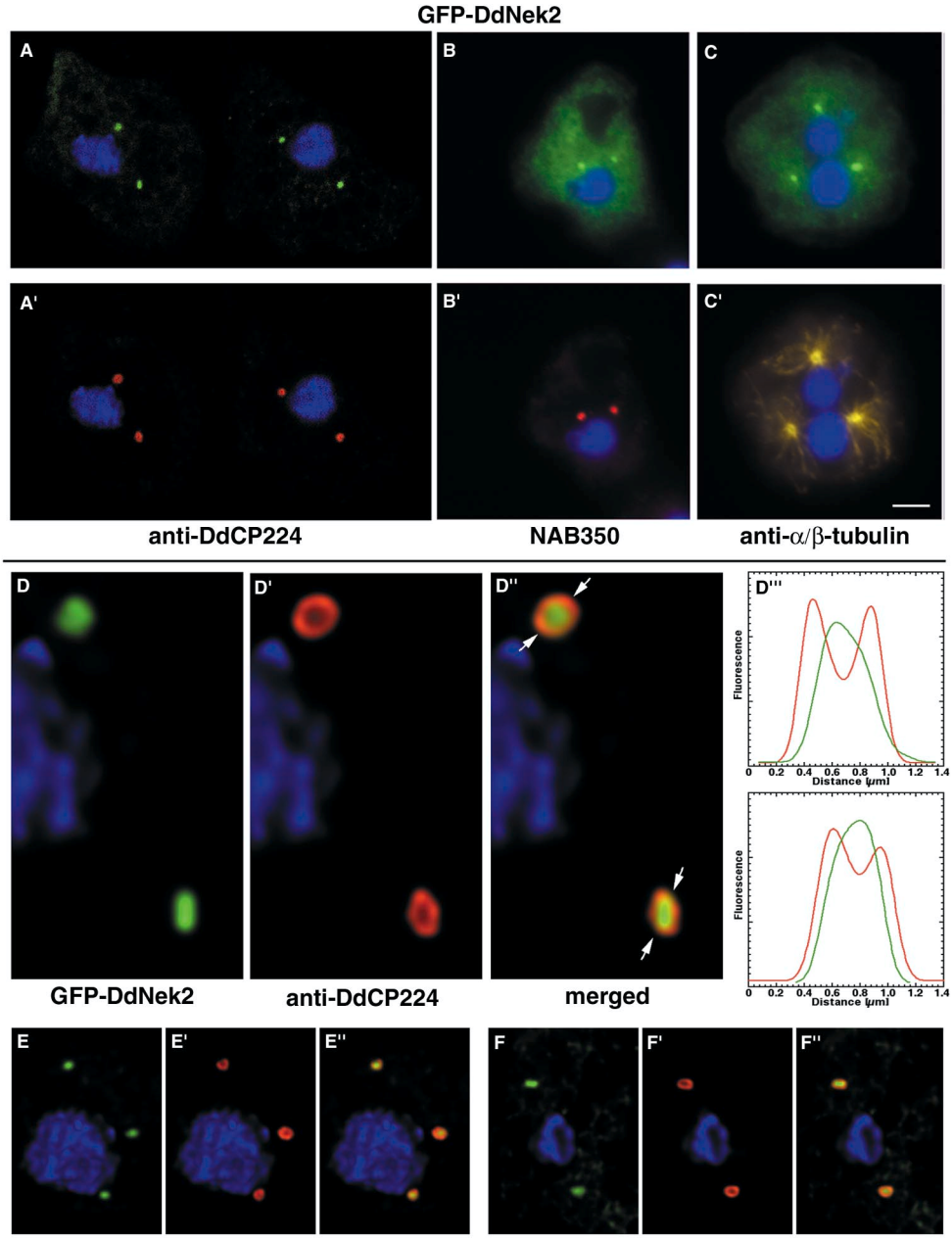

Fig. 6. Supernumerary MTOCs possess many features of centrosomes. Confocal (A,D,E,F) and non-confocal (B,C) immunofluorescence microscopy of cells fixed with methanol showing the presence of DdCP224 (A′), the NAB350 antigen (B′) and microtubules (C′′) at supernumerary MTOCs. The monoclonal antibody used to stain microtubules (shown in yellow for better visibility) in (C′) was 2/141 (Gräf et al., 1999). The secondary antibody was Cy3-labeled anti-mouse IgG. Nuclei were stained with DAPI (B,C) or with 2 µM TO-PRO3 (A,D,E,F) (Molecular Probes, Leiden, Nederlands) after a 1 hour treatment with 100 µg/ml RNaseA. (D-D′′) shows an enlarged image of the left cell in (A). Images (D,E,F) were processed with the Huygens 2.2 deconvolution program (Bilplane AG, Zürich, Switzerland). The brightest point projections of two (D,F) or three (E) confocal sections, respectively, are shown,. The merged images (D′′-F′′) are three examples demonstrating that DdCP224 labeling of the centrosomal corona (D′-F′) surrounds the structure labeled by GFP-DdNek2 (D-F) in both normal, nucleus-associated and supernumerary MTOCs. This is visualized graphically in (D′′′), where the fluorescence intensity of the DdCP224 (red) and GFP-DdNek2 (green) labeling, respectively, was plotted against the distance along a cross-section (arrows in D′′). Bar, 2 µm.

GFP-K33R cells (Fig. 7E-H). Thus, the supernumerary MTOCs, misshapen centrosomes, multiple GFP-foci and nuclear abnormalities were provoked by overexpression of the C-terminal domain. Furthermore, these results indicate the existence of two centrosomal targeting domains for DdNek2, one within the C-terminal domain and one within the catalytic or leucine zipper domain.

Discussion

Structural conservation of DdNek2

This work provides a characterization of DdNek2, the first NIMA-related kinase in a non-vertebrate organism, which is not only structurally related to mammalian Nek2 but also shares its centrosomal localization. The two protist kinases *Trypanosoma* Nrk and *Tetrahymena* Nrk (Gale and Parsons, 1993; Wang et al., 1998) are clearly related to NIMA, but there are no data supporting a centrosomal function. Among the non-vertebrate NIMA-related kinases where complete amino-acid sequences are available, *Drosophila* Nek2 (SPTREMBL accession no. Q9W3N8) is more likely to be a Nek2 homologue. However, its amino-acid sequence is almost twice as long as human and *Dictyostelium* Nek2 and, so far, a functional characterization of *Drosophila* Nek2 has not been published.

In vitro kinase assays revealed that the catalytic properties of bacterially expressed DdNek2 were very similar to those of human Nek2 expressed in insect cells. This is also reflected by similar structural properties, that is, both proteins are approximately the same length and have a catalytic domain that is followed by an unusual leucine zipper motif. In human Nek2, the leucine zipper motif consists of five heptad repeats with

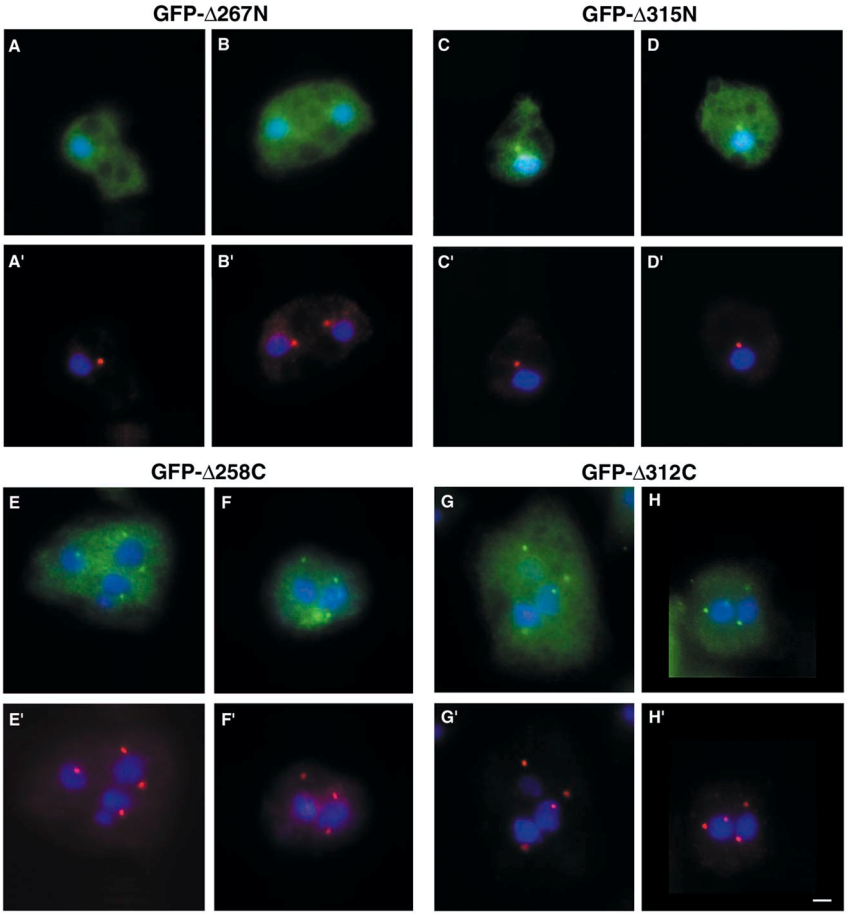

Fig. 7. Centrosomal localization and phenotypes of GFP-DdNek2 deletion mutants. Two representative examples each are shown for GFP-Δ267-N (A,B), GFP-Δ315-N (C,D), GFP-Δ258-C (E,F) and GFP-Δ312-C (G,H) mutants. Cells were fixed with methanol and labeled with anti-γ-tubulin. The secondary antibody was Alexa-568-labeled anti-rabbit IgG. Nuclei were stained with DAPI. Bar, 2 μm.

leucines at the *d* position, acidic instead of hydrophobic residues at the *a* and basic residues at the *g* position. Owing to this arrangement, the leucines are flanked by alternating basic and acidic residues within the α-helix (Fry et al., 1999). This seems to favor the formation of Nek2 homodimers via a very stable parallel coiled coil. Fry et al. (Fry et al., 1999) provided compelling evidence that autophosphorylation occurs at the C-terminal domain and requires dimerization and that each catalytic domain can trans-autophosphorylate the C-terminus. The leucine zipper motif of DdNek2 is longer than the usual four to five heptads. It extends over six heptad repeats and, if one accepts the isoleucine at position 314 as a conservative substitution for leucine, there are even eight heptads (amino-acid position 270-339; Fig. 1A). All *a* positions of these heptads are occupied by an acidic amino acid with the only exception being a tyrosine in the second heptad, and the *g* positions contain basic amino acids in five heptads and neutral ones (at physiological pH) in three heptads. Thus, the overall concept of the unusual Nek2 leucine zipper, which leads to dimerization, is evident in DdNek2 as well. This was supported by the kinase activity assays, which showed that the leucine zipper is required for enzymatic activity and that the C-terminal domain contains the target site for autophosphorylation. Therefore, at least some of the functions of the C-terminal domain seem to be conserved in *Dictyostelium* and humans, and it is likely that the tertiary structures of both proteins are comparable, although the amino-acid sequences of this region are only weakly conserved. However, a second coiled coil region followed by a cyclin A-type extended destruction box (D-box) at the very C-terminus of the vertebrate Nek2 proteins (Hames et al., 2001) is missing in DdNek2. This region also includes PEST-like motifs for rapid degradation (Rechsteiner and Rogers, 1996; Rhee and Wolgemuth, 1997; Uto et al., 1999). The A-type extended D-box seems to be responsible for the rapid degradation of *Xenopus* and mammalian Nek2A in early mitosis (Hames et al., 2001). DdNek2 does not contain this D-box, and there were no PEST sequences found using the PESTfind program. An investigation of cell-cycle-dependent changes of the DdNek2 expression

Table 1. Percentage of mutant phenotypes in GFP-DdNek2 and GFP-K33Rmutants exhibiting high expression of the GFP-fusion protein

	GFP-DdNek2 (*n*=316)	GFP-K33R (*n*=283)	GFP (control) (*n*=311)
1. One centrosome per nucleus	33.2%	33.4%	94.5%
2. Centrosomal and/or nuclear defects and one centrosome per nucleus	15.8%	22.9%	4.2%
3. Multiple GFP foci and one centrosome per nucleus	6.6%	9.7%	n.d.
4. Supernumerary MTOCs	27.8%	25.4%	1.3%
5. Supernumerary MTOCs and multiple GFP foci	17.1%	8.5%	n.d.

Cells with a centrosomal GFP fluorescence above a gray level of level 110 at a 256 level 8-bit scale (Hamamatsu CCD camera C5985, 1 second exposure, 100×NA1.3 lens) were evaluated (~25% of all cells in each clone). The five main categories of phenotype are listed in the left column. The first category represents normal cells. Among the cells of the second category, approximately two thirds exhibited deformed centrosomes and approximately one third exhibited signs, such as additional small DNA-masses (visible in DAPI stainings) or enlarged nuclei, of aneuploidy. The third category comprises cells with multiple GFP foci (characterized by the absence of γ-tubulin labeling). Many of these cells also possess deformed centrosomes (about two thirds) and signs of aneuploidy (about one fifth). Category four contains cells with supernumerary MTOCs (characterized by the presence of γ-tubulin); some cells showed signs of aneuploidy (~1/10). Cells in category five exhibit a combination of the phenotypes of categories three and four. Control cells transformed with the GFP-fusion vector without an insert showed that the mutant phenotypes were not caused by the transformation procedure or by expression of GFP alone.

level is not possible since *Dictyostelium* cells cannot be synchronized efficiently. Yet, the absence of PEST sequences and the A-type extended D-box suggests a high protein stability, similar to that of *Xenopus* Nek2B, which represents a more stable variant where these sequences are missing owing to alternative splicing (Uto and Sagata, 2000).

DdNek2 seems to be a component of the centrosomal core structure

None of the NIMA-related kinases in lower eukaryotes has so far been localized to the centrosome or spindle pole body. By contrast, using specific antibodies, DdNek2 was detected at the *Dictyostelium* centrosome during the entire cell cycle. Furthermore, it is a component of isolated centrosomes, which contain no microtubules (Gräf et al., 1998). Thus, DdNek2 is a genuine centrosomal component. Moreover, for the first time the localization of a Nek2 protein could be confirmed with a GFP-mutant. Deconvoluted confocal microscopy images clearly revealed that the GFP-DdNek2-labeled structure is surrounded by the corona and, thus, DdNek2 is likely to be the first known component of the centrosomal core structure in *Dictyostelium*.

DdNek2 is involved in formation of new MTOCs

The high amino-acid sequence similarity and similar localization pattern of DdNek2 and human Nek2 suggest comparable centrosomal functions. In human cells, overexpression of Nek2 causes centrosome splitting or centrosome dispersal (Fry et al., 1998b), suggesting two possibly independent functions in centrosome separation and centrosome maintenance. The former function is dependent on catalytic activity and presumably involves phosphorylation of C-Nap1, whereas the latter function is independent of catalytic activity since overexpression of catalytically inactive Nek2-K33R induces only centrosome dispersal. In *Xenopus* embryos, centrosome dispersal seems to be caused by Nek2B inhibition, since it was observed after injection of either mRNA encoding catalytically inactive Nek2B or inhibitory antibodies (Uto and Sagata, 2000). The dispersed centrosomal fragments contained γ-tubulin and were active as MTOCs. The occurrence of multiple GFP foci in some of the GFP-DdNek2 cells is independent of kinase activity and might be comparable to centrosome dispersal in human cells and frog embryos, but these GFP foci did not contain any *Dictyostelium* centrosome markers (not shown) and, thus, their significance remains elusive. Possibly they represent GFP-DdNek2 aggregates that are formed upon high overexpression of the fusion protein.

The supernumerary MTOC phenotype is the major phenotype of GFP-DdNek2, GFP-DdK33R, GFP-Δ258-C and GFP-Δ312-C mutants. It is clearly distinguished from the centrosome-dispersal and centrosome-splitting phenotype in human and *Xenopus* cells (Fry et al., 2000a; Fry et al., 1998b). First, in the *Dictyostelium* mutants, the normal nucleus-associated centrosome is still intact, and there are usually no more than two supernumerary MTOCs per nucleus. Second, the supernumerary MTOCs possess many features of normal, nucleus-associated centrosomes. They exhibit approximately the same size, seem to have a corona surrounding an inner core, include at least four centrosomal marker proteins and carry a microtubule aster. The observed nuclear aberrations in these mutants are most probably caused by supernumerary MTOCs, which are still associated with the nuclear envelope and interfere with chromosome segregation, unlike the majority of supernumerary MTOCs, which are free in the cytosol and have no access to the chromosome masses owing to the closed mitosis in *Dictyostelium*.

In human cells, Nek2 overexpression causes centrosome splitting but no increase in centrosome number, as an overdose of Nek2 seems to affect only cells in late S or G2, which have already duplicated their centriole pairs and, thus, have two centrosomal entities. Furthermore, this process seems to be strictly dependent on kinase activity (Fry et al., 1998b). By contrast, the presence of supernumerary MTOCs in DdNek2 mutants is clearly independent of catalytic activity and provoked by overexpression of the C-terminal domain alone. This observation may be explained by recent results obtained with *Xenopus* egg extracts. Fry et al. (Fry et al., 2000a) showed that *Xenopus* Nek2A plays a role in centrosome assembly: incubation of Nek2B-immunodepleted extracts with demembranated sperm nuclei retarded assembly of the pericentriolar matrix to the sperm basal body. Furthermore, Nek2B was one of the first proteins assembling to the nascent centrosome when spermheads were incubated with egg extracts. In correspondence to this Nek2B function, it could be that overexpression of the C-terminal domain in *Dictyostelium* induces assembly of centrosome components, finally resulting in the formation of complete centrosomes. Together with other cytosolic binding partners, the DdNek2 C-terminus could serve a seed function for the assembly of centrosomal components. This would explain the presence of DdNek2 in the center of supernumerary MTOCs and centrosomes. Alternatively, the overdose of C-terminal domain could competitively inhibit the interaction of endogenous DdNek2 with a binding partner required for proper centrosome duplication. This could result in centrosome/MTOC amplification. So far, no DdNek2-binding proteins are known, but in case of human Nek2 two interactors and substrates, C-Nap1 and protein phosphatase 1c (PP1c), have been identified by two-hybrid screening (Fry et al., 1998a; Helps et al., 2000). C-Nap1 is involved in centriole cohesion and dissociates from the centrosome after phosphorylation by Nek2, which seems to be a key step in centrosome separation (Fry et al., 1998a; Mayor et al., 2000). The role of a possible *Dictyostelium* homologue of C-Nap1 might be different owing to the structural divergence of human and *Dictyostelium* centrosomes and owing to the different mode and cell cycle stage of their duplication. The other Nek2 interactor, PP1c, regulates Nek2 and seems to be an effector of CDK1-cyclinB (Helps et al., 2000; Meraldi and Nigg, 2001). So far, it has been impossible to demonstrate an interaction of *Dictyostelium* PP1c (da Silva et al., 1999) with DdNek2 (A. M. da Silva and R. G., unpublished). This might be because of the fact that in humans PP1c interacts with the Nek2 C-terminal domain via the (R/K)(V/I)XF-motif (Helps et al., 2000), which is conserved in most PP1c-binding proteins but not in DdNek2.

DdNek2 possesses at least two centrosome-binding domains

At least two centrosomal DdNek2-interacting proteins can be

postulated, since the localization of GFP-DdNek2 deletion constructs revealed the existence of at least two independent centrosomal targeting domains. The first resides in the C-terminal domain because the corresponding GFP-Δ312-C mutant clearly localized to normal and supernumerary MTOCs. The second is present in the complementary part of DdNek2, comprising the catalytic and leucine zipper domain, which is represented by GFP-Δ315-N. Since the GFP-Δ267-N mutant, which is devoid of the leucine zipper, failed to localize to the centrosome, the centrosomal binding domain could be a part of the coiled coil domain. However, for sterical reasons, it is hard to imagine that the leucine zipper, which seems to be necessary for dimerization of Nek2 proteins, serves a further function as a centrosomal targeting domain. It appears more likely that the centrosome-binding determinant lies within the catalytic domain that can only interact with its centrosomal binding partner as a dimer. Owing to the similarities with DdNek2, it seems likely that human Nek2 contains two centrosomal targeting domains as well. A myc-tagged deletion mutant (Nek2ΔLZ), which cannot dimerize and has the C-terminal domain fused directly to the catalytic domain, still localized to centrosomes (Fry et al., 1999), but this could be mediated alone by a dimerization-independent C-terminal targeting domain as in DdNek2.

Conclusions

DdNek2 is the first centrosomal NIMA-related kinase identified in a non-vertebrate species, and it is targeted to the centrosome by two independent determinants. Both DdNek2 and vertebrate Nek2 proteins seem to be involved in the assembly of MTOCs; however, the exact functions reflected by the phenotypes of Nek2 mutants in *Dictyostelium* and vertebrates could be different. The role in MTOC assembly is independent of catalytic activity. Certainly there should also be a DdNek2 function requiring catalytic activity, which could be related to the role of vertebrate Nek2 in centrosome separation. However, as most of the protein is found in the cytosol, this could also be a cytosolic function, which does not affect centrosome duplication or mitosis. Conversly, Nek2 of mammals and *Xenopus* may have unknown tasks in the centrosome duplication cycle that are independent of catalytic activity. In the future, a much broader spectrum of Nek2 functions can be expected from the identification and analysis of new binding partners and from the continuation of the comparative study of Nek2 proteins in higher animals and lower eukaryotes.

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References

Adams, I. R. and Kilmartin, J. V. (2000). Spindle pole body duplication: a model for centrosome duplication? *Trends Cell Biol.* **10**, 329-335.

Blaauw, M., Linskens, M. H. and van Haastert, P. J. (2000). Efficient control of gene expression by a tetracycline-dependent transactivator in single *Dictyostelium discoideum* cells. *Gene* **252**, 71-82.

Bollag, D. M., Rozycki, M. D. and Edelstein, S. J. (eds) (1996). *Protein Methods.* New York: Wiley-Liss, Inc.

da Silva, A. M., Zapella, P. D., Andrioli, L. P., Campanha, R. B., Fiorini, L. C., Etchebehere, L. C., da-Costa-Maia, J. C. and Terenzi, H. F. (1999). Searching for the role of protein phosphatases in eukaryotic microorganisms. *Braz. J. Med. Biol. Res.* **32**, 835-839.

Daunderer, C., Schliwa, M. and Gräf, R. (1999). *Dictyostelium discoideum*: a promising centrosome model system. *Biol. Cell* **91**, 313-320.

Daunderer, C., Schliwa, M. and Gräf, R. (2001). *Dictyostelium* centrin-related protein (DdCrp), the most divergent member of the centrin family, possesses only two EF-hands and dissociates from the centrosome during mitosis. *Eur. J. Cell Biol.* **80**, 621-630.

Euteneuer, U., Gräf, R., Kube-Granderath, E. and Schliwa, M. (1998). *Dictyostelium* γ-tubulin: molecular characterization and ultrastructural localization. *J. Cell Sci.* **111**, 405-412.

Fry, A. M. and Nigg, E. A. (1997). Characterization of mammalian NIMA-related kinases. *Methods Enzymol.* **283**, 270-282.

Fry, A. M., Schultz, S. J., Bartek, J. and Nigg, E. A. (1995). Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of *Aspergillus nidulans*. *J. Biol. Chem.* **270**, 12899-12905.

Fry, A. M., Mayor, T., Meraldi, P., Stierhof, Y. D., Tanaka, K. and Nigg, E. A. (1998a). C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J. Cell Biol.* **141**, 1563-1574.

Fry, A. M., Meraldi, P. and Nigg, E. A. (1998b). A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *EMBO J.* **17**, 470-481.

Fry, A. M., Arnaud, L. and Nigg, E. A. (1999). Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. *J. Biol. Chem.* **274**, 16304-16310.

Fry, A. M., Descombes, P., Twomey, C., Bacchieri, R. and Nigg, E. A. (2000a). The NIMA-related kinase X-Nek2B is required for efficient assembly of the zygotic centrosome in *Xenopus laevis*. *J. Cell Sci.* **113**, 1973-1984.

Fry, A. M., Mayor, T. and Nigg, E. A. (2000b). Regulating centrosomes by protein phosphorylation. *Curr. Top. Dev. Biol.* **49**, 291-312.

Gale, M., Jr and Parsons, M. (1993). A *Trypanosoma brucei* gene family encoding protein kinases with catalytic domains structurally related to Nek1 and NIMA. *Mol. Biochem. Parasitol.* **59**, 111-121.

Gräf, R. (2001a). Isolation of centrosomes from *Dictyostelium*. *Methods Cell Biol.* **67**, 337-357.

Gräf, R. (2001b). Maltose-binding protein as a fusion tag for the localization and purification of cloned proteins in *Dictyostelium*. *Anal. Biochem.* **289**, 297-300.

Gräf, R., Brusis, N., Daunderer, C., Euteneuer, U., Hestermann, A., Schliwa, M. and Ueda, M. (2000a). Comparative structural, molecular and functional aspects of the *Dictyostelium discoideum* centrosome. *Curr. Top. Dev. Biol.* **49**, 161-185.

Gräf, R., Daunderer, C. and Schliwa, M. (1999). Cell cycle-dependent localization of monoclonal antibodies raised against isolated *Dictyostelium* centrosomes. *Biol. Cell* **91**, 471-477.

Gräf, R., Daunderer, C. and Schliwa, M. (2000b). *Dictyostelium* DdCP224 is a microtubule-associated protein and a permanent centrosomal resident involved in centrosome duplication. *J. Cell Sci.* **113**, 1747-1758.

Gräf, R., Euteneuer, U., Ueda, M. and Schliwa, M. (1998). Isolation of nucleation-competent centrosomes from *Dictyostelium discoideum*. *Eur. J. Cell Biol.* **76**, 167-175.

Hames, R. S., Wattam, S. L., Yamano, H., Bacchieri, R. and Fry, A. M. (2001). APC/C-mediated destruction of the centrosomal kinase Nek2A occurs in early mitosis and depends upon a cyclin A-type D-box. *EMBO J.* **20**, 7117-7127.

Helps, N. R., Luo, X., Barker, H. M. and Cohen, P. T. (2000). NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. *Biochem. J.* **349**, 509-518.

Kalt, A. and Schliwa, M. (1996). A novel structural component of the *Dictyostelium* centrosome. *J. Cell Sci.* **109**, 3103-3112.

Kay, R. R. and Williams, J. G. (1999). The *Dictyostelium* genome project: an invitation to species hopping. *Trends Genet.* **15**, 294-297.

Kochanski, R. S. and Borisy, G. G. (1990). Mode of centriole duplication and distribution. *J. Cell Biol.* **110**, 1599-1605.

Levedakou, E. N., He, M., Baptist, E. W., Craven, R. J., Cance, W. G., Welcsh, P. L., Simmons, A., Naylor, S. L., Leach, R. J., Lewis, T. B. et al. (1994). Two novel human serine/threonine kinases with homologies to the cell cycle regulating *Xenopus* MO15, and NIMA kinases: cloning and characterization of their expression pattern. *Oncogene* **9**, 1977-1988.

Lu, K. P. and Hunter, T. (1995). The NIMA kinase: a mitotic regulator in *Aspergillus nidulans* and vertebrate cells. *Prog. Cell Cycle Res.* **1**, 187-205.

Lupas, A., VanDyke, M. and Stock, J. (1991). Predicting coiled coils from protein sequences. *Science* **252**, 1162-1164.

Mack, G. J., Rees, J., Sandblom, O., Balczon, R., Fritzler, M. J. and Rattner, J. B. (1998). Autoantibodies to a group of centrosomal proteins in human autoimmune sera reactive with the centrosome. *Arthritis Rheum.* **41**, 551-558.

Mann, S. K. O., Devreotes, P. N., Eliott, S., Jermyn, K., Kuspa, A., Fechheimer, M., Furukawa, R., Parent, C. A., Segall, J., Shaulsky, G. et al. (1998). Cell biological, molecular genetic, and biochemical methods used to examine *Dictyostelium*. In *Cell Biology: A Laboratory Handbook*, vol. 1 (ed. J. E. Celis), pp. 431-465. San Diego: Academic Press.

Mayor, T., Stierhof, Y. D., Tanaka, K., Fry, A. M. and Nigg, E. A. (2000). The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. *J. Cell Biol.* **151**, 837-846.

Meraldi, P. and Nigg, E. A. (2001). Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. *J. Cell Sci.* **114**, 3749-3757.

Moens, P. B. (1976). Spindle and kinetochore morphology of *Dictyostelium* discoideum. *J. Cell Biol.* **68**, 113-122.

Morio, T., Urushihara, H., Saito, T., Ugawa, Y., Mizuno, H., Yoshida, M., Yoshino, R., Mitra, B. N., Pi, M., Sato, T. et al. (1998). The *Dictyostelium* developmental cDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. *DNA Res.* **5**, 335-340.

Moudjou, M., Bordes, N., Paintrand, M. and Bornens, M. (1996). γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. *J. Cell Sci.* **109**, 875-887.

O'Connell, M. J., Norbury, C. and Nurse, P. (1994). Premature chromatin condensation upon accumulation of NIMA. *EMBO J.* **13**, 4926-4937.

Osmani, S. A., Pu, R. T. and Morris, N. R. (1988). Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. *Cell* **53**, 237-244.

Osmani, A. H., O'Donnell, K., Pu, R. T. and Osmani, S. A. (1991). Activation of the nimA protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the bimE checkpoint. *EMBO J.* **10**, 2669-2679.

Paintrand, M., Moudjou, M., Delacroix, H. and Bornens, M. (1992). Centrosome organization and centriole architecture: their sensitivity to divalent cations. *J. Struct. Biol.* **108**, 107-128.

Paoletti, A., Moudjou, M., Paintrand, M., Salisbury, J. L. and Bornens, M. (1996). Most of centrin in animal cells is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles. *J. Cell Sci.* **109**, 3089-3102.

Pu, R. T., Xu, G., Wu, L., Vierula, J., O'Donnell, K., Ye, X. S. and Osmani, S. A. (1995). Isolation of a functional homolog of the cell cycle-specific NIMA protein kinase of *Aspergillus nidulans* and functional analysis of conserved residues. *J. Biol. Chem.* **270**, 18110-18116.

Rechsteiner, M. and Rogers, S. W. (1996). PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* **21**, 267-271.

Rhee, K. and Wolgemuth, D. J. (1997). The NIMA-related kinase 2, Nek2, is expressed in specific stages of the meiotic cell cycle and associates with meiotic chromosomes. *Development* **124**, 2167-2177.

Roos, U. P. (1975). Mitosis in the cellular slime mold *Polysphondylium violaceum*. *J. Cell Biol.* **64**, 480-491.

Schultz, S. J. and Nigg, E. A. (1993). Identification of 21 novel human protein kinases, including 3 members of a family related to the cell cycle regulator nimA of *Aspergillus nidulans*. *Cell Growth Differ.* **4**, 821-830.

Schultz, S. J., Fry, A. M., Sutterlin, C., Ried, T. and Nigg, E. A. (1994). Cell cycle-dependent expression of Nek2, a novel human protein kinase related to the NIMA mitotic regulator of *Aspergillus nidulans*. *Cell Growth Differ.* **5**, 625-635.

Tanaka, K., Parvinen, M. and Nigg, E. A. (1997). The in vivo expression pattern of mouse Nek2, a NIMA-related kinase, indicates a role in both mitosis and meiosis. *Exp. Cell Res.* **237**, 264-274.

Ueda, M., Schliwa, M. and Euteneuer, U. (1999). Unusual centrosome cycle in *Dictyostelium*: correlation of dynamic behavior and structural changes. *Mol. Biol. Cell* **10**, 151-160.

Uto, K. and Sagata, N. (2000). Nek2B, a novel maternal form of Nek2 kinase, is essential for the assembly or maintenance of centrosomes in early *Xenopus* embryos. *EMBO J.* **19**, 1816-1826.

Uto, K., Nakajo, N. and Sagata, N. (1999). Two structural variants of Nek2 kinase, termed Nek2A and Nek2B, are differentially expressed in *Xenopus* tissues and development. *Dev. Biol.* **208**, 456-464.

Wang, S., Nakashima, S., Sakai, H., Numata, O., Fujiu, K. and Nozawa, Y. (1998). Molecular cloning and cell-cycle-dependent expression of a novel NIMA (never-in-mitosis in *Aspergillus nidulans*)-related protein kinase (TpNrk) in Tetrahymena cells. *Biochem. J.* **334**, 197-203.

Weber, I., Gerisch, G., Heizer, C., Murphy, J., Badelt, K., Stock, A., Schwartz, J. M. and Faix, J. (1999). Cytokinesis mediated through the recruitment of cortexillins into the cleavage furrow. *EMBO J.* **18**, 586-594.

Wetterauer, B. W., Salger, K., Carballometzner, C. and Macwilliams, H. K. (1995). Cell-density-dependent repression of discoidin in *Dictyostelium discoideum*. *Differentiation* **59**, 289-297.

Wetterauer, B., Morandini, P., Hribar, I., Murgia-Morandini, I., Hamker, U., Singleton, C. and MacWilliams, H. K. (1996). Wild-type strains of *Dictyostelium discoideum* can be transformed using a novel selection cassette driven by the promoter of the ribosomal V18 gene. *Plasmid* **36**, 169-181.

Wu, L., Osmani, S. A. and Mirabito, P. M. (1998). A role for NIMA in the nuclear localization of cyclin B in *Aspergillus nidulans*. *J. Cell Biol.* **141**, 1575-1587.

Ye, X. S., Xu, G., Pu, R. T., Fincher, R. R., McGuire, S. L., Osmani, A. H. and Osmani, S. A. (1995). The NIMA protein kinase is hyperphosphorylated and activated downstream of p34cdc2/cyclin B: coordination of two mitosis promoting kinases. *EMBO J.* **14**, 986-994.