Identification of Proteins That Interact with the Central Coiled-Coil Region of the Human Protein Kinase NEK1†

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ABSTRACT: NEK protein kinases are evolutionarily conserved kinases structurally related to the *Aspergillus nidulans* mitotic regulator NIMA. At least nine members of the NEK family in vertebrates have been described to date, but for most of them the interacting protein partners are unknown. The pleiotropic deleterious effects and the formation of kidney cysts caused by NEK1 mutation in mice emphasize its involvement in the regulation of diverse cellular processes and in the etiology of polycystic kidney disease (PKD), respectively. Here we report the identification of proteins that interacted with the human NEK1 protein kinase in a yeast two-hybrid screen of a human fetal brain cDNA library, using the catalytic and regulatory domains of NEK1 separately as baits. These proteins are known to take part either in the development of PKD, in the double-strand DNA break repair at the G2/M transition phase of the cell cycle, or in neural cell development. The proteins involved in PKD include the motor protein KIF3A and the proteins tuberin and α-catulin. Mapping studies of the human NEK1 regulatory domain (NRD) indicated a strong interaction of most of the proteins retrieved from the library with putative coiled coils located in the central region of NRD. Our results give further support to the previous observation that NEK1 is of functional importance for the etiology of PKD.

The eukaryotic cell cycle is highly organized and is regulated by the concerted action of protein kinases and phosphatases. Among the many protein kinase families described, members of the *N*IMA-*r*elated *k*inases (NEKs)[1] take part in the cell cycle and constitute the kinase family less well characterized functionally (*1*). As a founder member of the family, NIMA (*n*ever *i*n *m*itoses, gene *A*) was identified in *Aspergillus nidulans* as a serine/threonine protein kinase critical for the transition between checkpoint G2 and mitosis (G2/M) of the cell cycle (*2*, *3*). Overexpression of wild-type NIMA results in chromatin condensation and premature mitotic arrest in mammalian cells (*4*).

Protein kinases homologous to NIMA have been described containing about 40% identity at their N-terminal catalytic domain, but almost none at their C-terminal, which constitutes their regulatory domain and might be involved in determining their specificity for substrate(s) or interactions with other proteins.

The mouse NEK1 (mNEK1) is mainly expressed in germ cells (*5*), and the study of mutant mice *kat* and *kat*^{2J} (*6*) established that the gene coding for mNEK1 is mutated in both cases and is directly involved in the etiology of PKD, besides causing pleiotropic deleterious effects in those mutant mice, including male sterility.

The key feature for substrate specificities of the NIMA/NEK family of protein kinases is a preference for a large hydrophobic residue at the −3 position relative to the serine/threonine residue phosphorylated, although this preference may vary according to the family member (*7*). Only NEK-2, -8, and -11 have their functions analyzed in more detail. NEK2 has a cell cycle-dependent expression peaking at the beginning of mitosis and has been shown to associate to centrosomes leading to their disruption, which is necessary for the transition G2/M (*8*). These events are the result of phosphorylation of the centrosome-associated protein c-NAP1 (*9*). NEK8 has its expression maximum at G0, and a yeast two-hybrid screen indicated BICD2 as its interacting protein, a human protein homologous to the *Drosophila melanogaster* protein bicaudal D present in the cytoskeleton and microtubules (*1*). Although not all NEK members influence the cell cycle, the most recently discovered member of the family, NEK11, has been implicated in DNA replication and genotoxic stress responses, but its substrates and interacting proteins have not been determined yet (*10*).

In this report we describe the importance of the human NEK1 (hNEK1) internal coiled-coil region in the interaction with proteins recovered from a yeast two-hybrid screen and involved in PKD, double-strand DNA (dsDNA) break repair at the G2/M transition phase of the cell cycle, and neural cell developmental stages in vertebrates.

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[1] Abbreviations: ATRX, alpha thalassemia/mental retardation syndrome X-linked; 53BP1, p53 binding protein 1; dsDNA, double-strand DNA; FEZ1, fasciculation and elongation protein zeta 1 (zygin I); FEZ2, fasciculation and elongation protein zeta 2 (zygin II); KIF3A, kinesin 3A; MRE11, meiotic recombination 11 homologue A; NEK1, NIMA-related kinase 1; NIMA, never in mitosis, gene A; NKD, NEK1 kinase domain; NRD, NEK1 regulatory domain; PKD, polycystic kidney disease; PP2A, protein phosphatase 2A; ZBRK1, zinc finger and BRCA1-interacting protein with a KRAB domain.

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MATERIALS AND METHODS

Human NEK1 cDNA Sequence. The full-length hNEK1 cDNA (DKFZp564L2416Q3; GenBank accession number AL050385) was kindly provided by the Resource Center/Primary Database (Berlin, Germany). This clone has been isolated from a human fetal brain cDNA library (DKFZhfb2) created by Stefan Wiemann (DKFZ, Heidelberg, Germany).

Yeast Two-Hybrid Screen and Sequence Analysis. The yeast two-hybrid screen (*11*) of a human fetal brain cDNA library (Clontech) was done with the yeast strain L40 [trp1-901, his3Δ200, leu2-3, ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lac GAL4] using the kinase domain (NKD, amino acids 1−250) and the regulatory domain (NRD, amino acids 251−1258) of hNEK1 separately as a bait fused to the yeast LexA DNA-binding domain in the vector pBTM116 (*12*).

Oligonucleotides KD-S (5′-CGGAATTCCATATGGAGAAGTATGTTAGACTAC) and KD-AS (5′-CGGGATCCTTAGCGTTTGGCTATAAAACCTTTCTC) with *Eco*RI and *BamH*I restriction sites, respectively, were used to amplify the sequence coding for the kinase domain by PCR prior to the insertion in pBTM116. Oligonucleotides RD-S (5′-CAAGGATCCGCATTGAAAAGTTTCTCTCTC) and RD-AS (5′-CAAGGATCCTTATTCATCATTATCTTCTTG) with *Bam*HI restriction sites were used for the amplification of the sequence coding for the regulatory domain of the hNEK1 by PCR prior to its insertion in pBTM116.

Yeast cells transformation was done according to the protocol supplied by Clontech. The screening was done in minimal medium plates without tryptophan, leucine, and histidine and containing 10 mM 3-amino-1,2,4-triazole (3-AT). Complementary DNAs of positive clones obtained from the library were sequenced with a DNA sequencer, model 377 (Perkin-Elmer/Applied Biosystems). Prediction for coiled-coil structures (*13*) for hNEK1 and for the proteins that interacted with hNEK1 after yeast two-hybrid system screening was performed by the software available at the web site www.ch.embnet.org/software/COILS_form.html of the Swiss Institute for Experimental Cancer Research. Phosphorylation site prediction was performed by the software NetPhos 2.0 Prediction Server available at the web site of the Center for Biological Sequence Analysis in Denmark (*14*) at www.cbs.dtu.dk/services/NetPhos/.

Cloning of hNEK1 Partial Sequences for Yeast Two-Hybrid System Analysis. Partial nucleotide sequences of the hNEK1 coding for the kinase domain, regulatory domain, and its parts containing regions prone to formation of coiled-coil structures were inserted in vector pBTM116 for further analysis of protein−protein interactions using the yeast two-hybrid system. The combination of oligonucleotides KD-S/KD-AS and RD-S/RD-AS was used for the PCR amplification of the sequences coding for the kinase and regulatory domains of hNEK1, respectively. The NRD domain was divided into four regions, whereby NRD1 includes the first two coiled coils, NRD2 includes coiled coils 3 and 4, and NRD3 includes coiled coil 5. NRD4 includes NRD1 and NRD2 regions (Figure 1B). NRD1 nucleotide sequence was amplified using oligonucleotides RD1-S (5′-CGGGATCCCATCCATATTGGAGAAAGG) and RD1-AS (5′-CGGGATCCTCAAGAAAAAGATGATGGAGC), NRD2 using oligonucleotides RD2-S (5′-CGGGATCCCAGGACAGTATGAACATTAC) and RD2-AS (5′-CGGGATCCTCATGTTCTTTTAGTACAGC), NRD3 using oligonucleotides RD3-S (5′-CGGGATCCCAGAGGCTTATGAGAGAG) and RD3-AS (5′-CGGGATCCTCATTCATCATTATCTTCTTG), and NRD4 using oligonucleotides RD1-S and RD2-AS. The amplified fragments were cloned in the *Bam*HI restriction site in vector pBTM116 (bait vector), and the orientation and frame of each insert were confirmed by restriction endonuclease analysis and sequencing. In a similar approach the cDNAs encoding the NEK1 fragments NRD, NRD1, NRD2, NRD3, and NRD4 were subcloned into the "prey vector" pGAD424 (Clontech).

Assay for β-Galactosidase Activity in Yeast Cells. β-Galactosidase activity in yeast cells was measured by the filter assay method. Yeast transformants (Leu^+, Trp^+, His^+) were transferred onto nylon membranes, permeabilized in liquid nitrogen, and placed on Whatman 3MM paper previously soaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM $MgCl_2$, 50 mM 2-mercaptoethanol, pH 7.0) containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). After incubation at 37 °C for 30 min to 1 h, depending on the clone, the yeast cells forming dark blue colonies were taken from replica plates for further analysis.

Protein Expression. The nucleotide sequences in the library vector pACT2 between restriction sites *Eco*RI and *Xho*I coding for the proteins retrieved after yeast two-hybrid system screening were inserted in vector pProExHTb (Invitrogen) for expression of recombinant His-tagged proteins in *Escherichia coli* BL21 cells. NEK1 nucleotide sequences coding for NRD1, NRD2, NRD3, and NRD4 regions were inserted in vector pGEX5x2 (Amersham Pharmacia Biotech) for expression of recombinant GST-tagged proteins in *E. coli* BL21 cells. Only FEZ1, FEZ2, NRD2, and NRD3 could be recovered as soluble proteins for in vitro analyses after 4 h induction of protein synthesis in *E. coli* strain BL21 at 30 °C using 0.4 mM IPTG. Soluble human His-tagged receptor activator C-kinase protein (RACK1) (*15*) was used for controls in in vitro protein−protein interaction assays.

In Vitro Binding Assay and Western Blot Analysis. Free GST, GST-NRD2, and GST-NRD3 proteins were allowed to bind to 20 μL of glutathione−Sepharose 4B resin (Amersham Pharmacia) in 1 mL of total protein extract in PBS for 1 h at 4 °C. After incubation, the beads saturated with recombinant proteins were washed three times with PBS 1× (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4 °C. About 20 μg of recombinant 6×His-RACK1, 6×His-FEZ1, and 6×His-FEZ2 fusion proteins previously purified by affinity chromatography were separately added to the resins containing GST or GST fusion protein and incubated in 0.1 mL of PBS 1× for 4 h at 4 °C to allow for protein−protein interaction. The beads were then washed three times with 0.5 mL of PBS 1×, followed by three washes with 0.5 mL of PBS 1× containing 0.1% Triton X-100 and three washes with 0.5 mL of PBS 1×. Resin-bound protein samples were resolved in a SDS−12% polyacrylamide gel in duplicate. After electrophoresis the proteins were transferred to a PVDF membrane by semidry electroblotting. After saturation with unspecific protein (5% bovine serum albumin) in TBS 1× (0.15 M NaCl, 20 mM Tris-HCl, 0.05% Tween-20, pH 7.2) the membranes were incubated with a mouse anti-His tag (1:3000) or mouse anti-GST antiserum (1:3000) for 1 h each. After three washes with TBS 1×/0.05% Tween-20, the membranes were incu-

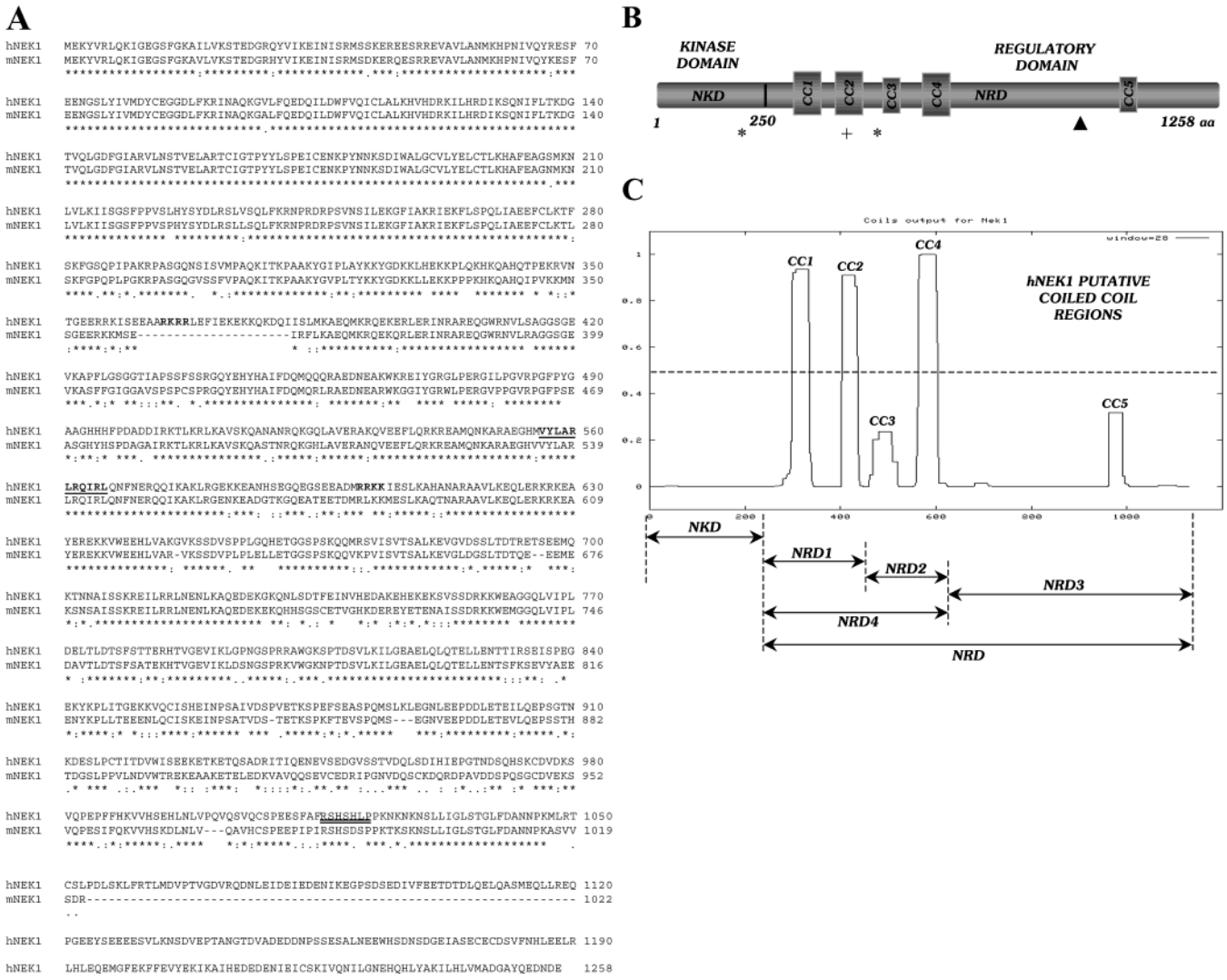

FIGURE 1: (A) Protein sequence alignment of human and mouse NEK1. Alignments were generated using the Clustalw program (*51*) at http://www.ebi.ac.uk/clustalw. Gaps are introduced to maximize the alignment with identical residues (*), conservative substitutions (:), and semiconservative substitutions (.) indicated. The putative nuclear localization signals RKRR and RRKK are in bold face, the putative nuclear export signal VYLARLRQIRL between them is in bold face and underlined, and the 14-3-3 protein recognition motif RSHSHLP at the C-terminus is double underlined. GenBank accession numbers are as follows: hNEK1, AL050385; mNEK1, XP_146362. (B) Scheme representing the primary sequence of human NEK1 with its kinase and regulatory domains, nuclear localization signals (*), nuclear exclusion signal (+), 14-3-3 protein recognition motif (▲), and putative coiled-coil regions CC1−CC5. (C) Human NEK1 protein sequence analyzed by the COILS program using a window of 28 amino acids. The propensity of a sequence to form coiled coils is given on a 0−1 vertical scale plotted as a function of the linear amino acid sequence. For further analysis, coils were numbered 1−5 for cDNA constructions used to express proteins corresponding to the four segments NRD1, NRD2, NRD3, and NRD4.

bated with secondary HRP-conjugated rabbit anti-mouse IgG antibody (1:5000; Santa Cruz Biotech) for 1 h and washed again three times with TBS 1×. The membranes were developed by chemiluminescence (luminol reagent; Santa Cruz Biotech) for detection of 6×His-tagged and GST fusion proteins.

RESULTS

Characteristics of hNEK1 Primary and Secondary Structures. The nucleotide sequence from clone DKFZp564L-2416Q3 (AL050385) codes for a protein of calculated mass 128.57 kDa and isoelectric point pI = 5.58. Figure 1 shows that the kinase domain is located at the N-terminus and includes the first ~250 amino acid residues. Homology to other NEK family members is restricted to this domain. Compared to mNEK1 (XP_146362), hNEK1 has 236 extra amino acids at its C-terminus. Two putative nuclear localization signals (NLS, bold face in Figure 1A and * in Figure 1B) are deduced, comprising amino acid residues 364−367 (RKRR) and 601−604 (RRKK) (*16*). One putative nuclear export signal (NES, bold face and underlined in Figure 1A and + in Figure 1B) comprises amino acids 556−566 (VYLARLRQIRL) (*17*), and a putative 14-3-3 protein recognition sequence is present, comprising amino acids 1016−1022 (RSHSHLP, double underlined in Figure 1A and ▲ in Figure 1B) (*18*), whereby the probability of Ser1019 being phosphorylated is 96.2% according to analysis with the program NetPhos 2.0 (*14*).

Identification of Proteins That Interact with hNEK1. To identify proteins interacting with the hNEK1, we employed the yeast two-hybrid system (*12*) and screened a human fetal brain cDNA library. The hNEK1 kinase (NKD) and regulatory (NRD) domains were tested separately as baits in two screens. A total of about 1.5 × 10^6 transformants for each

Table 1: Human NEK1-Interacting Proteins Identified by the Yeast Two-Hybrid System Screen

protein interacting with hNEK1	bait	insert length (bp)[a]	coded protein residues (retrieved/complete sequence)	domain composition (native protein)[b]	function[c]	ref
ZBRK1	NKD	798	1−267/532	KRAB domain C2H2 Zn finger domains	corepressor of GADD45 expression	*19*
FEZ-1	NRD	1170	124−392/392	coiled-coil region	neuronal development	*43*
FEZ-2	NRD	970	130−325/325	coiled-coil region	neuronal development	*43*
14-3-3 protein (YWHAH)	NRD	750	1−250/250	coiled-coil region	cell cycle regulator	*18*
ATRX (Rad54 homologue)	NRD	2800	1−960/973	coiled-coil region	dsDNA break repair	*38*
MRE11	NRD	750	100−350/708	coiled-coil region	homologous recombination	*49*
53BP1	NRD	3000	450−1400/1972	BRCT domain	dsDNA break repair	*37*
KIF3A	NRD	2100	1−702/702	coiled-coil region	motor transport	*50*
PP2A subunit B56	NRD	2000	59−602/602		cell cycle regulator	*41*
tuberin	NRD	1300	1325−1763/1763	coiled-coil region	determination of polycystin-1 membrane localization	*29*
α-catulin (α-catenin-like)	NRD	2000	60−734/734	coiled-coil region	signal transduction	*30*

[a] Aproximate length of the sequences retrieved from the library. [b] Other domains may be present. [c] Other functions may be known.

screen was plated, and the clones were grown on selective minimal medium plates (without tryptophan, leucine, and histidine). Cells from grown colonies showing a strong blue color in the subsequent β-galactosidase filter assay had their plasmid DNAs extracted and sequenced. Using the NKD as bait, the only clone retrieved had a sequence coding for the N-terminal half of ZBRK1 (amino acid residues 1−267), a corepressor protein that interacts with BRCA1 inhibiting the transcription of *GADD45*, a tumor suppressor gene (*19*). Ten different proteins were identified using the NRD as bait (Table 1). These can be divided into three groups: (i) proteins associated with the PKD, (ii) proteins that take part in the dsDNA repair during the G2/M transition phase of the cell cycle, and (iii) proteins taking part in the regulation of neural cell development and function.

The first group includes the motor transport protein KIF3A (GenBank accession number Q9Y496), the signaling protein tuberine (NP_066400), and a protein homologous to α-catulin, an α-catenin-like protein (NP_003789). The second group consists of ATRX, a RAD54 homologue (NP_0033556), a protein homologous to 53BP1 (Q12888), MRE11A (NP_005590), PP2A regulatory subunit B56 (NP_006236), and a 14-3-3 protein member (YWHAH, NP_003396). The third group includes FEZ1 (Q99689) and its homologous protein FEZ2 (NP_005093), as well as KIF3A again.

Most of these proteins are prone to form coiled-coil regions frequently involved in protein−protein interactions, whereby the protein homologous to 53BP1 and ATRX show only about 50% probability of coiled-coil formation. Table 1 summarizes the domain organization and functional characteristics of the proteins found to interact with hNEK1.

Phosphorylation Site Prediction. The substrate specificities for NIMA (*20*, *21*) and for NEK6 (*7*) have been determined and revealed a new and unique nature of substrate recognition by this group of protein kinases. The recognition site includes a phenylalanine or other hydrophobic residue at position −3 relative to the phosphorylated Ser/Thr residue.

Table 2 shows peptide sequences taken from the proteins retrieved in our screening containing Ser/Thr residues with a probability of phosphorylation higher than 70% and a hydrophobic (Phe) residue at the −3 position relative to the phosphorylation site. The hNEK1 has many Ser/Thr residues

Table 2: Putative Phosphorylation Sites Present in Human NEK1 and Its Interacting Proteins[a]

protein interacting with hNEK1	residue no.	sequence	theoretical probability of phosphorylation by NIMA (%)
ZBRK1	Ser 181	KFPASQKLI	97.5
FEZ-1	Ser 335	TFGSSGTDK	87.6
FEZ-2	Ser 102	LFDTSDDEE	76.3
ATRX	Ser 394	EFIASDSDE	97.9
(RAD54 homologue)	Ser 597	LFIISTKAG	83.3
MRE11	Thr 659	IFPTTSKTP	92.9
	Ser 689	DFESSEDDD	99.2
53BP1	Thr 373	AFRSTPFIV	87.6
	Ser 898	SFCESSSET	97
	Ser 1068	HFPSSQGEE	98.8
KIF3A	Ser 148	LVRVSYLEI	98.6
	Ser 676	SLRQSLMKL	94.6
PP2A subunit B56	Ser 109	ALKDSPTQE	97.6
tuberin	Ser 641	VVRFSPYCV	99.3
	Thr 927	SFDDTPEKD	95.5
α-catulin	Ser 389	KISHSLNEL	91.3
hNEK1	Thr 782	SFSTTERHT	95.1
	Tyr 1206	FFEVYEKIK	82.3

[a] The phosphorylated residue (Ser/Thr) and the Phe residue at −3 (or another hydrophobic residue) are underlined. Two putative autophosphorylation sites in human NEK1 are also indicated.

in its primary structure with a hydrophobic residue at the −3 position with a high probability of phosphorylation. This suggests that these residues might be targets of NEK1 autophosphorylation.

Mapping of hNEK1 Domains Involved in Protein−Protein Interactions. Sequences coding for different regions of hNEK1 (Figure 1C) were inserted into pBTM116 and assayed against the proteins retrieved from the fetal human brain cDNA library after yeast two-hybrid screening to map the NEK1 protein regions involved in the interaction (Figure 2). Also, these sequences were assayed against themselves to test if hNEK1 engages in dimer formation (Figure 3).

The NKD domain interacted strongly with ZBRK1, PP2A, and tuberin and weakly with α-catulin, while the NRD domain interacted with all proteins, including ZBRK1, PP2A, tuberin, and α-catulin (Figure 2B). Interestingly, construct NRD4, which contains coiled coils 1−4, and construct NRD2, which encompasses coiled coils 3 and 4 only, also

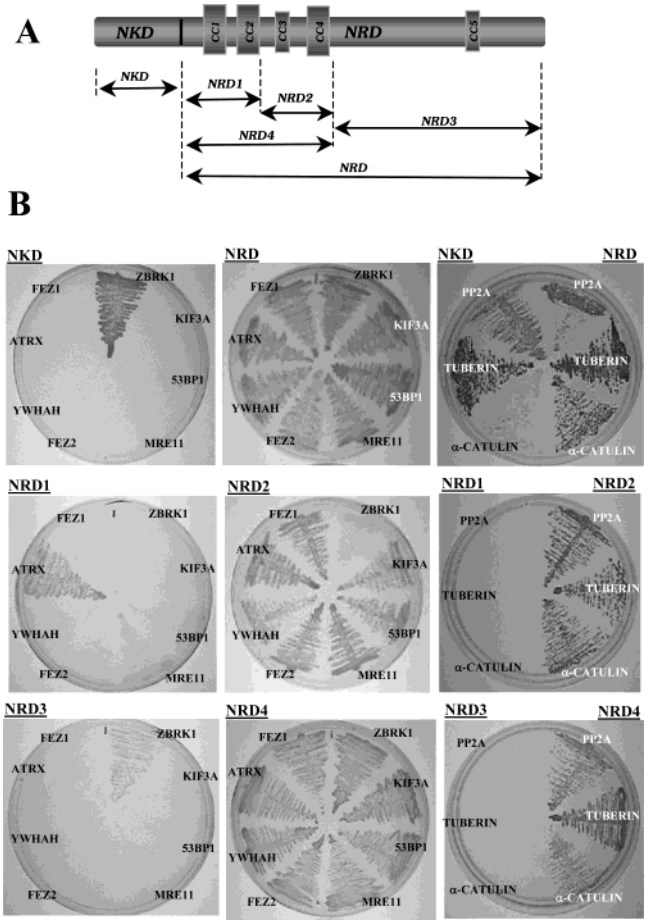

FIGURE 2: (A) Schematic representation showing the different regions of human NEK1 used for mapping the interactions with the proteins retrieved from the library after a yeast two-hybrid screen. (B) Interaction of human NEK1 regions with the proteins retrieved after screening the fetal human brain cDNA library with the NEK1 kinase and regulatory domains separately as baits. Bait (underlined) and prey plasmids (indicated on the plates) containing the sequences coding for the different proteins were cotransfected into yeast strain L40, and protein−protein interactions were evaluated by the ability of the cells to grow on minimal medium lacking tryptophan, leucine, and histidine and containing 10 mM 3-AT. The presence of both plasmids in the L40 cells was checked by growth on plates containing histidine but lacking tryptophan and leucine (not shown). Transformants grown on these plates always stained blue after 1 h incubation in the β-galactosidase filter assay (not shown).

interacted with all proteins retrieved. In contrast, construct NRD1 which contains coiled coils 1 and 2 interacted strongly with ATRX and weakly with MRE11 and 53BP1. NRD3 which contains putative coiled coil 5 showed a significant interaction with ZBRK1 only (Figure 2B).

NKD interacted with constructs NRD and NRD3, but NRD interacted only with NRD3 (Figure 3B). This seems to suggest that the region NRD3 could be relevant for a possible NEK1 dimerization, which might be stabilized by contacts of NKD, since NRD does not interact with NRD alone. Interestingly, NRD3 interacted with NRD2, and both NRD2 and NRD4 also interacted strongly with NRD3. Together, these data suggest an antiparallel orientation of the two NEK1 monomers in a putative NEK1 dimer, where the C-terminal side (NRD3) of one NEK1 monomer is in contact with the N-terminal side (NRD2/4) of the other NEK1 monomer (Figure 3A). In accordance with this, NKD interacts with

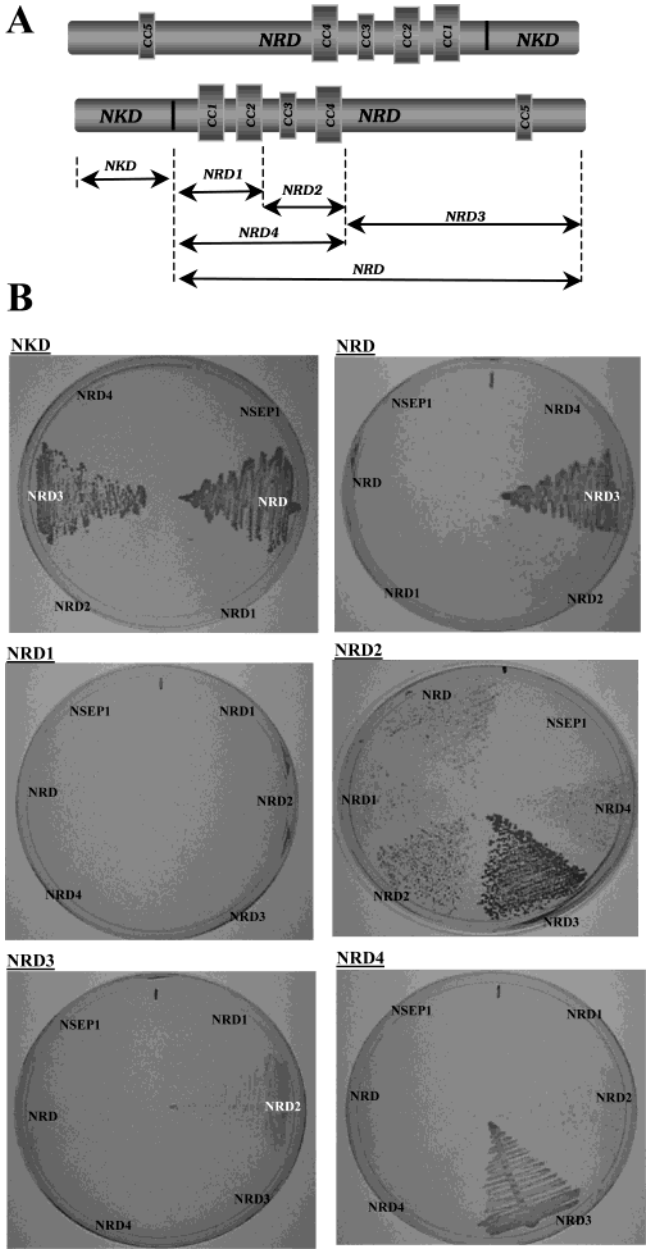

FIGURE 3: (A) Schematic representation showing the different regions of human NEK1 used for mapping the Nek1 regions involved in dimer formation. A second NEK1 molecule is shown to visualize the putative antiparallel orientation of the two monomers. Such an arrangement would be in agreement with the interaction data as identified by the yeast two-hybrid analysis. (B) Interaction of NEK1 regions with itself. Bait (underlined, vector pBTM116) and prey plasmids (indicated on the plates, vector pGAD424) coding for the indicated regions of NEK1 were cotransfected into yeast strain L40, and protein−protein interactions were evaluated by the ability of the cells to grow on minimal medium lacking tryptophan, leucine, and histidine and containing 10 mM 3-AT. The presence of both plasmids in the L40 cells was checked by growth on plates containing histidine but lacking tryptophan and leucine (not shown). Transformants grown on these plates always stained blue after 1 h incubation in the β-galactosidase filter assay (not shown).

NRD3, and adjacent regions in the NRD structure interact with each other; i.e., NRD2 interacted with NRD3 (strongly), NRD4 (weakly), NRD (weakly), and with itself (strongly). None of the NEK1 constructs showed interaction with NRD1 alone. A construct in vector pACT2 containing the coding sequence for the nonrelated nuclease-sensitive element

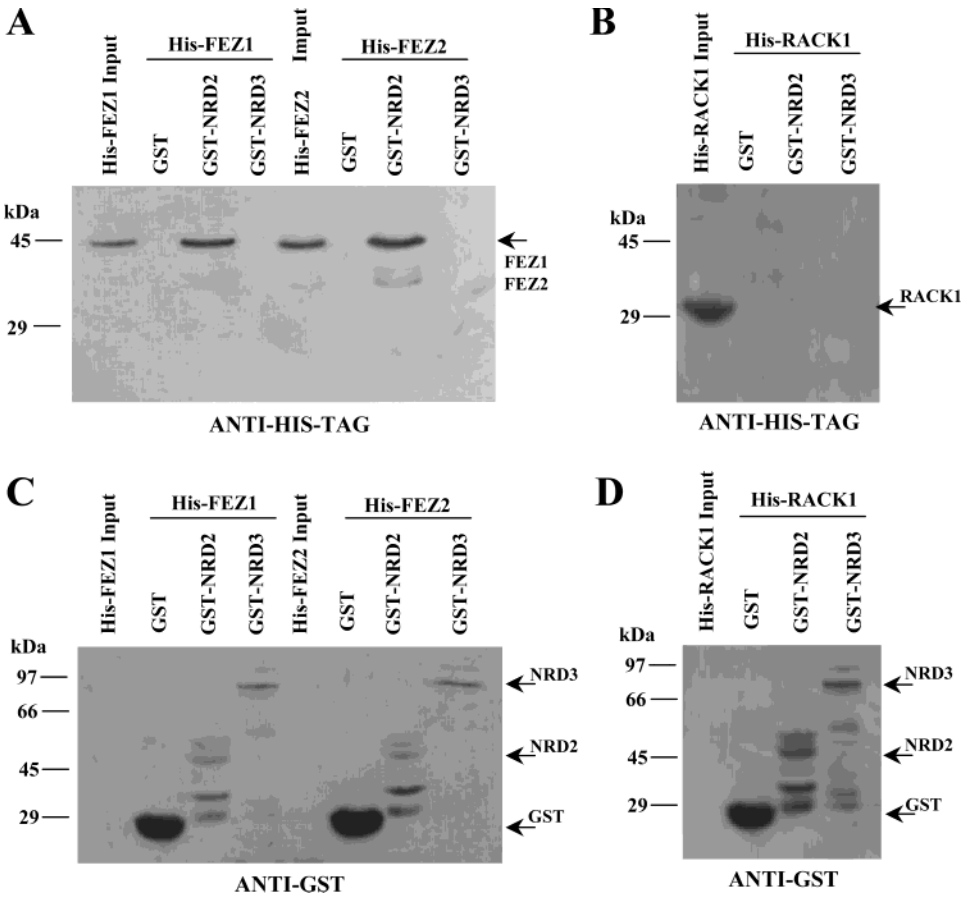

FIGURE 4: (A) In vitro binding assays of the interactions between regions NRD2 and NRD3 from human NEK1 and proteins FEZ1 and FEZ2 retrieved from the human fetal brain cDNA library. Both 6×His-FEZ1 and 6×His-FEZ2 interacted strongly with GST-NRD2 but neither with GST-NRD3 (nor with free GST), confirming the results obtained by yeast two-hybrid system analysis. (B) In vitro binding assay of the interaction between regions GST-NRD2 and GST-NRD3 from hNEK1 and 6×His-RACK1, where no interaction occurs under the conditions tested. (C) and (D) show the same membranes as in (A) and (B), respectively. This time an anti-GST antibody was used to develop the membrane to demonstrate equal loading of the beads with GST or GST fusion proteins used for the pull-down assays shown in (A) and (B).

binding protein NSEP1 (*22*) was used as control, and none of the proteins coded by the different pBTM116 constructs interacted with Gal4AD-NSEP1 (Figure 3B).

In Vitro Assays of the Interaction of NRD2 and NRD3 with FEZ1, FEZ2, and RACK1. The interaction of hNEK1 regions NRD2 and NRD3 with proteins FEZ1 and FEZ2 retrieved from the human fetal brain cDNA library after screening was confirmed using an in vitro pull-down assay with partially purified recombinant fusion proteins (GST or 6×His-tag fusions: e.g., His-FEZ1) expressed in *E. coli* BL21 (Figure 4). GST-NRD2 interacted specifically with both His-FEZ1 and His-FEZ2, whereas control protein GST alone did not (Figure 4A). The number of washings indicates a strong interaction among these proteins. GST-NRD3, which had not interacted with either FEZ1 or FEZ2 in the yeast two-hybrid assays, also did not interact in vitro with FEZ1 and FEZ2 (Figure 4A). There was no interaction of constructs GST-NRD2 and GST-NRD3 with the nonrelated control fusion protein 6×His-RACK1 (*15*) under the conditions tested (Figure 4B). The equal loading of the glutathione−Sepharose 4B beads with GST or GST fusion proteins was controlled by developing the Western blot with an anti-GST antibody (Figure 4C,D). The other proteins that interacted with hNEK1 were not tested because they could not be obtained so far in soluble form.

DISCUSSION

We performed a yeast two-hybrid screen with the human protein kinase NEK1, which is involved in the etiology of PKD, to identify putative substrate proteins or other interacting proteins that might regulate NEK1 or may be regulated by NEK1. We identified 11 different proteins that have previously been described to be involved in the etiology of the PKD, with DNA repair during the G2/M transition state of the cell cycle, or with neuronal cell development.

The search for intracellular protein partners for NEK family members is ongoing since the characterization of NIMA from *A. nidulans* as a key protein regulating the progression of the cell cycle at the G2/M transition phase and increased after the confirmation that homologous proteins are also associated with this process in vertebrates (*4*).

In light of the etiology of PKD we found it especially interesting that we retrieved two proteins, KIF3A and tuberin, that have been recently associated with the development of PKD when the genes encoding these proteins are affected by mutations.

The manifold functions performed by kinesin family members include such distinct processes as transporting various cargoes, organizing the cytoskeleton, and controlling signal transduction (*23*), all of which are prone to phosphorylation control by protein kinases. KIF3A, a kinesin family

protein, is expressed ubiquitiously but predominantly in brain, testis, and adrenal medulla (*24*) and is important for the determination of the embryonic body plan, particularly for establishment of its laterality (*25*). The facial dysmorphism and male sterility in *kat* mutant mice having no functional mNEK1 (*6*) could be related to a lack of KIF3A phosphorylation. The involvement of NEK1 in a signaling pathway that regulates transport functions in the kidney has been proposed (*6*), and Lin and co-workers recently demonstrated the importance of KIF3A mutations in the etiology of PKD (*26*).

The tumor suppressor protein tuberin is encoded by the *TSC2* gene and plays a critical role as a regulator of cell growth and proliferation (*27*). Together with *TSC1*, the gene coding for the protein hamartin, it is involved in an autosomal dominant disorder known as tuberous sclerosis complex when one of these two genes is affected by mutations (*28*). A functional link between PKD and tuberin was also identified (*29*), since tuberin determines the correct membrane localization of polycystin-1.

The most relevant fact involving the identification of a member of this protein family in our analysis is that polycystin-1, the main protein related to the PKD etiology (*30*), interacts with the complex E-caderin/catenin in the membrane (*31*). We found α-catulin, an α-catenin-like protein, as a NEK1 interacting protein in our yeast two-hybrid screen. It can thus be speculated that hNEK1 is a member of the polycystin-1-related signaling pathway, which has already been suggested elsewhere (*6*).

Although NEK1 shows a high expression in meiotic germ cells (*5*), the pleiotropic effects caused by *nek1* gene mutations in mice (*6*) affected also the central nervous system. Furthermore, in situ RNA hybridization analysis had shown high levels of NEK1 expression in distinct regions of the nervous system (*32*). These facts prompted us to screen a human fetal brain cDNA library using NEK1 as bait. Several of the proteins that interacted with hNEK1 are already known to take part in processes associated with the repair of double-strand DNA breaks at the G2/M transition phase. Among these, MRE11 is required for the mammalian DNA double-strand break end-joining reaction (*33*) and takes part in homologous recombination in *Saccharomyces cerevisiae* (*34*), and 53BP1 is a mediator of the DNA damage checkpoint (*35*). Besides, 53BP1 from *Xenopus laevis* is associated with chromatin (*36*) and colocalizes with MRE11 after irradiation (*37*). Considering that the breast cancer protein 1 (BRCA1) interacts with MRE11 during dsDNA repair (*38*) and ZBRK1 interacts with BRCA1 (*19*), hNEK1 could be involved in the regulation of ZBRK1 function possibly by phosphorylating it and thus influencing the interaction BRCA1/ZBRK1. ATRX, another protein found in our screening, is homologous to RAD54, which is important for the repair of dsDNA breaks by homologous recombination (*39*), contributing to the maintenance of genomic stability in vertebrate cells (*40*). PP2A, protein phosphatase 2A, is a Ser/Thr phosphatase with a high expression in brain, and it is implicated in the negative control of cell growth and division (*41*).

The *Caenorhabditis elegans* UNC-76 homologue human protein FEZ1 is involved in axonal outgrowth and fasciculation as shown by complementation of *unc-76 C. elegans* mutants (*42*). Phosphorylation of the membrane-associated form of FEZ1 by protein kinase C isoform zeta leads to its migration from the membrane to the cytosol, where its function remains unknown (*43*). There are many proteins containing coiled-coil regions taking part in signaling networks and growth control regulation via protein−protein interactions. Except for the corepressor protein ZBRK1, retrieved from the library using NKD as bait, and PP2A, all other proteins retrieved in our screen show a significant probability to form coiled-coil structures, although for 53BP1 and ATRX it is lower than 50%. These proteins interacted preferentially with the NRD2 region of hNEK1, including the third and fourth predicted coiled coils in its structure (Figure 2). Such coiled-coil interactions are usually strong (*44*), and our results are in agreement with such a strong interaction, since all clones were retrieved from a library screen in the presence of 10 mM 3-AT.

Taken together, our interaction domain mapping studies suggest that the NEK1 interacting proteins can be divided into three major groups. The first consists of proteins that do interact with both NKD and NRD (ZBRK1, tuberin, α-catulin, PP2A). Interestingly, ZBRK1 was retrieved with NKD as bait but later also shown to interact with NRD. For the other three proteins of this group, the opposite is true: they were first found to interact with NRD, and subsequently their interaction with NKD could also be confirmed. The second group consists of proteins that only interact with NRD1, NRD2, and NRD4 (ATRX, MRE11, 53BP1). The third group finally interacts only with NRD2 and NRD4 but not with NRD1 (FEZ1, FEZ2, KIF3A, and YWHAH).

The control of interaction with distinct substrates via specific coiled-coil interaction mediated by the different NRD regions may be relevant to other NEK family members, as each has a regulatory domain with low homology relative to the other members of the family. Most interestingly, c-NAP1 and BICD2, proteins that interacted with NEK2 and NEK8, respectively, also form coiled coils, although the coiled-coil region of NEK2 is not required for the interaction with c-NAP1 (*45*).

Previous studies for the identification of substrate specificity determinants for NIMA protein kinase using synthetic peptides indicated that Phe-Arg-X-Ser/Thr represents its optimal sequence for phosphorylation (*20*). Considering the kinase domain homology among NEK family members, it is reasonable to speculate that the phosphorylation site sequence Phe-X-X-Ser/Thr (*46*) is also present in their substrates, i.e., that they may have Phe or another hydrophobic residue at the −3 position relative to the phosphorylated Ser/Thr residue, while the preference of each family member for a specific hydrophobic residue may vary (*7*). Of all proteins here reported, only KIF3A has no sequence containing a Ser/Thr residue with more than 70% probability of phosphorylation and a phenylalanine at position −3 relative to potential phosphorylation sites, but many containing Ser/Thr residues with hydrophobic residues smaller than Phe (e.g., Val) at position −3 and Arg at position −2, characteristics also important for NIMA phosphorylation (Table 2). The fact that hNEK1 itself has many phosphorylation sites conforming to these attributes suggests its putative autophosphorylation. Recently, Feige and co-workers (*47*) reported the characterization of a new protein, Nurit, retrieved from a yeast two-hybrid screen of a rat testis cDNA library using the first 506 amino acid residues including the

mutated catalytic domain and the first two coiled-coil regions of NEK1 as bait. Nurit has the potential to form three coiled-coil regions that could be involved in the interaction with NEK1. As a protein kinase that causes pleiotropic effects when mutated, the regulation of NEK1 activity must be tightly controlled. This could be achieved by interaction with regulator proteins such as 14-3-3 (*48*). HNEK1 has a putative sequence in its C-terminus (Figure 2) recognized by this group of proteins and interacted with one of its members in our yeast two-hybrid system analysis. The fact that NRD3, which contains the putative 14-3-3 binding site, does not interact with the 14-3-3 protein YWHAH in the yeast might be due to the lack of phosphorylation of Ser1019 of the protein produced in the yeast cells.

To our knowledge, this is the first report where proteins known to take part in the development of PKD or the dsDNA break repair at the G2/M transition phase of the cell cycle are shown to interact with hNEK1, paving a new way for further studies involving the regulation of G2/M transition by hNEK1, as well as detailed studies of the interaction of hNEK1 with the proteins KIF3A and tuberin which have previously been shown to be affected by mutations that occur in the course of the development of PKD.

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