Nek9, a Novel FACT-associated Protein, Modulates Interphase Progression*

Received for publication, October 20, 2003, and in revised form, November 25, 2003
Published, JBC Papers in Press, December 19, 2003, DOI 10.1074/jbc.M311477200

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The heterodimeric Spt16-Pob3/DUF/FACT complex is a class of chromatin structure modulators with important roles in replication and transcription. Although regarded as a transcription elongator for chromatin template, little is known about the mode of action and involvement in other molecular processes of the mammalian FACT. Here we report the identification of a novel interacting and functional partner of FACT, Nek9. Nek9 forms a stable, ~600-kDa complex with FACT in the interphase nuclei. Its active form is characterized by phosphorylation-dependent electrophoretic mobility shift and phosphorylation at a conserved residue within the activation loop (Thr210). When complexed with FACT, Nek9 exhibits markedly elevated phosphorylation on Thr210. Cell cycle analysis on the Nek9dsRNAi cells directly implicated Nek9 in maintaining proper G1 and S progression, a role temporally correlated to the formation of a phosho-Nek9-FACT complex. Collectively, these observations provide evidence that Nek9, potentially as an active enzymatic partner of FACT, mediates certain FACT-associated cellular processes, which are ultimately essential for interphase progression.

The human Spt16 and SSRP1 proteins constitute an abundant, nuclear, and heterodimeric complex called FACT. FACT was initially discovered as a chromatin-specific elongation factor that facilitates transcription of chromatin templates in vitro (1, 2). The exact mechanism by which FACT aids the movement of RNA polymerase II along nucleosomal DNA is not known. By interacting with histone H2A/H2B dimers, FACT has been postulated to render intranucleosomal histone connections more flexible (3).

The genes that encode these two FACT polypeptides and, more significantly, the existence of such stable heterodimeric factors are highly conserved among eukaryotes. Homologous heterodimers have been purified and identified from yeast and frog cells. The Xenopus DNA unwinding factor (DUF)1 complex is also composed of Spt16 and SSRP1 homologs (4). Unlike the mammalian counterpart, DUF was directly implicated in DNA replication, as immunodepletion of this complex led to a reduced ability to replicate exogenously added sperm nuclei or plasmid DNA. However, its ability to alter nucleosomal DNA structure is conserved (5). Studies on the corresponding Spt16-Pob3 heterodimer in yeast have provided the most extensive up-to-date evidence on the pleiotropic nature of this heterodimer as well as its role as a chromatin structure modulator. Mutations in either genes cause various allele-specific defects in transcription initiation and elongation (6–8) and DNA replication (9, 10). Additionally, conditional mutants exhibit stalled progression in G1 and S phases (10, 11), perhaps as indirect consequences of misregulated chromatin processes. Consistently, genetic and physical interactions were observed between Spt16-Pob3 and a myriad of transcription or replication proteins, such as transcription elongator Paf1 complex (12, 13), histone acetyltransferase complex NuA3 (14), histone deacetylase Rpd3 (9), general transcription factor TFIIE (15), putative chromatin factors San1 (16) and Chd1 (13, 17), Nhp6 (a high mobility group protein that forms the SPN complex with the heterodimer) (9), DNA polymerase α (10, 18), and various DNA replication factors (10, 11, 18). Collectively, this assortment of observations directly links FACT/DUF/Spt16-Pob3 to replication and transcription and, more specifically, to the structural regulation of chromatin, as such an entity serves as template for both processes.

The existing evidence is consistent with the notion that FACT and homologous complexes possess a single central activity that is required for the progression of replication and transcription. However, their roles at multiple steps within these two processes may be mediated by differential coordination with various factors. The potential existence of such an interaction network is substantiated by the aforementioned group of ySpt16-Pob3-interacting proteins. Hence, to further delineate the role of human FACT in chromatin-related cellular processes, our research efforts have been focused on the identification and functional characterization of its interacting proteins. By generating monoclonal antibodies directed against SSRP1, and subsequently performing antibody affinity purification, we were able to isolate FACT-associated protein complexes from HeLa nuclear extracts. One of the polypeptides identified in such search is the Nek9 protein.

Nek9 (previously termed Nek8) was originally identified as a β-casein kinase that associates with a putative substrate Bicd2 (19). It was independently identified as a Nek6- and Ran GTPase-binding protein under a different name, Nerc1 (20). Based on kinase domain sequence similarity, Nek9 was a recent addition to the NIMA-like kinase (Nek) family, whose dozen or so members have diverse but relatively unknown functions. In addition to the N-terminal catalytic domain, Nek9 contains a central, RCC1-like, seven-repeats region, followed by a coiled-coil domain. Despite the presence of a nuclear localization signal, previous publications have reported a cyto-

*This work was supported by National Science Council Grants NSC90-2321-B002-003 and the Institute of Biological Chemistry, Academia Sinica, Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: DUF, DNA unwinding factor; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; ds, double-stranded; FACs, fluorescence-activated cell sorting; mAb, monoclonal antibody.

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plasmic localization of this kinase. Furthermore, Nek9 is putatively involved in the microtubule dynamics and has been implicated in the regulation of mitotic progression (20).

Experimental results presented in this report confirm the presence of a nuclear form of Nek9. One of the major supporting factors is the in vitro interaction between Nek9 and FACT, the characterization of which was done using biochemical and immunocytochemical methods. The activation loop residue Thr210 was phosphorylated by immunocytochemical methods. A better mechanistic understanding of which was done using biochemical and molecular methods. Thus, our data collectively suggest that Nek9, by virtue of forming an active kinase complex with FACT, may be involved in FACT-mediated functional pathways, the compromise of which would ultimately lead to impeded interphase progression.

EXPERIMENTAL PROCEDURES

Molecular Cloning of the Nek9 cDNA—DNA library was synthesized from poly(A)+ RNA of HeLa cells. The complete open reading frame containing cDNAs were obtained from this library by PCR using LA Taq DNA polymerase (Takara) with synthetic primers 5'-CCGGCATGGTCGTTGCGAGGATGACG-3' (neak-forward) and 5'-TGGGAGACGACCTGCTAGACCCAGC-3' (neak-reverse, without the stop codon). The −3-kb fragment was subsequently subcloned into TOPO-TA plasmid (Invitrogen) for sequence verification and further cloning. C-terminally Flag-tagged Nek9 was produced by inserting a Not-SalI fragment from the above Nek9-TA plasmid into the pCMV-Tag4A mammalian expression vector pVL1393 (Pharmingen). For bacterial expression of recombinant Nek9, two partial coding sequences (NotI-EcoRI and BamHI-EcoRI corresponding to amino acids 1–266 and 291–559, respectively) were ligated to pET-32 prokaryotic expression plasmid (Novagen).

Preparation of Recombinant Proteins and Antibodies—The expression plasmids pVL-Nek9-FLAG, pVL-Nek9-Myc, pVL-His6-SSRP1, and pVL-FLAG-FACT140 were introduced into Sf9 insect cells using a BaculoGold transfection kit (Pharmingen). The recombinant baculoviruses were produced and harvested according to the manufacturer’s instructions. The Nek9-FLAG protein was purified by anti-FLAG (M2) immunoprecipitation (Invitrogen) and eluted with synthetic FLAG peptide (0.5 molar). His-tagged Nek9s (amino acids 1–266 and 291–559), HisSSRP1, and His-FACT140 were expressed in BL21 (Lys) cells and purified from the clarified lysates on nickel-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer’s instructions. Monoclonal antibodies against SSRP1 (2B12, 3E4, 7F8, and 10D1) and FACT140 (8D2) were produced in mice using the respective recombinant proteins. Anti-Nek9 rabbit antigen, termed αNek9-N and αNek9-M, were raised by independent injections with His-Nek9-(1–266) and His-Nek9-(291–559), respectively, and subsequently affinity-purified through Nek9-FLAG column. Antisera against phosphorylated Nek9 activation loop peptide were generated in rabbits using keyhole limpet hemocyanin-conjugated phosphorylated peptides (Nek9-206–216, BRAETV GVTPTY, phosphorylated at Thr210). The antisera from immunized rabbits were affinity-purified successively with bacterial His-Nek9-conjugated phosphorylated peptides, columns. Both anti-Nek9 and Thr210-Nek9 phospho-specific rabbit antibodies were produced by Daye (Taiwan).

Immunoprecipitation and Western blot Analysis—Cells were rinsed with PBS. Whole cell extracts were obtained using a buffer containing 20 mM HEPES, pH 7.4, 0.2 mM NaCl, 0.5% Triton X-100, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES, 10 mM Na2-VO3, 1 mM NaF, 1 mM dithiothreitol, plus protease inhibitors. Protein concentration was determined by the Bradford reagent (Bio-Rad). Immunoprecipitations were done with the indicated antibodies prebound to protein G-Sepharose (Amersham Biosciences) and washed in the cell lysis buffer containing 0.3 mM NaCl. Similar conditions apply to the in vitro precipitations, which were done in 25 mM HEPES, pH 7.4, 0.1 mM Triton X-100, 0.4 mM NaCl, 10% glycerol, and protease inhibitors. Nuclear extracts in the amount of 40 mg in a volume of 5 ml were loaded onto a precalibrated Sephacryl S-400 HR column with a bed volume of 135 ml (Amersham Biosciences). Gel filtration was done in the same buffer at a flow rate of 0.5 ml/min. Ninety fractions were collected at 1.5 ml intervals. Upon fractionation, proteins present in each fraction were precipitated with 11.2 ml of methanol and 11.2 ml of ice-cold 100% trichloroacetic acid. Recovered samples were run on SDS-PAGE and analyzed by Western blotting using the indicated antibodies. For detection of FACT-associated complexes, immunoprecipitation was carried out with 10D1 on equal amounts of the selected fractions. Precipitated polypeptides were electrophotographically separated and analyzed by Western blotting.

Indirect Immunofluorescence and Confocal Microscopy—All steps of the immunostaining procedure were done at room temperature. HeLa cells were first washed with PBS twice followed by fixation with fresh 2% paraformaldehyde in PBS for 15 min. After a brief rinse in PBS, cells were permeabilized with 0.5% Triton X-100 in PBS, blocked with 1% bovine serum albumin (BSA) in PBS, and probed with primary antibodies. Secondary antibody incubation was done for 1 h using Alexa 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG (Molecular Probes, Inc.). To visualize DNA, cells were counter-stained with DAPI. For detection of DNA replication sites, HeLa cells on coverslips were pulse-labeled with 10 µM BrdUrd at 37 °C for 30 min. Upon fixation and permeabilization, cells were subjected sequentially to acid treatment and trypsin enzymatic digestion before being stained (mouse anti-BrdUrd, Sigma). Stained cells were analyzed with the Zeiss LSM-510 inverted confocal laser-scanning microscope, using a 63×/NA 1.4 oil immersion objective lens.

Cell Culture, Transfection, and Cell Cycle Synchronization—All HeLa cells unless otherwise stated were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin. Cells were transfected using LipofectAMINE (In-vitrogen) according to the manufacturer’s instructions. To establish a plasmid-based dsRNAi system targeting endogenous Nek9, annealed oligonucleotides corresponding to partial Nek9 sequence were designed and ligated to the pSilencer 1.0-U6 vector (Ambion) according to the manufacturer’s instructions. The cDNA sequence of the targeted Nek9 mRNA region is 5′-GACCATCGTCTTAAAAGCC-3′ (nucleotides 2193–2211). The same sequence in the inverted orientation was used as the nonspecific dsRNAi control. Generation of cell lines stably harboring dsRNAi was done by first co-transfecting HeLa cells with pcDNA3 (Invitrogen) and the indicated pSilencer 1.0-U6 vectors. Positive clones were selected subsequent to a 14-day culture in the presence of 1 mg/ml G418 and eventually maintained with 0.2 mg/ml G418.

For M phase synchronization, subconfluent cells were treated with 200 ng/ml nocodazole for 10 h. Mitotic cells were harvested through detachment by mitotic shake-off. Cells remaining on the flasks after shake-off were harvested by trypsinization and regarded as G2 cells. Cells were synchronized at the G2/S boundary by double thymidine block. Subconfluent culture was blocked with 2 mM thymidine (final) and incubated for 19 h. Cells were washed three times with PBS and then incubated with fresh serum-rich medium for 10 h. Subsequently, 10 mM thymidine lasting for 30 min was added, and the cells were released by extensive washes with PBS. For determination of active DNA replication, immediately before harvest at each time point, cells were pulse-labeled with 10 mM BrdUTP for 15 min. After harvest by trypsinization, cells were fixed with ethanol (70% final), denatured in 2 N HCl for 30 min at 37 °C, and subsequently enzymatically treated with RNase A. Cell cycle profiles and degree of BrdUrd labeling were monitored by FACS.

Colonies Formation Assay—Different clones of both the wild-type and Nek9knockdown cells were seeded into 6-cm diameter tissue culture dishes at 600 or 1200 cells per dish, in triplicate, and grown in the presence of 100 µg/ml G418. Dishes were incubated for 10–12 days in a humidified incubator. Colonies were subsequently fixed with methanol and stained with 1% crystal violet.

Fluorescence-activated Cell Sorting (FACS) Analysis—Control and Nek9knockdown cells, after being released from the double thymidine block or mitotic shake-off, were collected by trypsinization at the indicated time.
Immunocomplexes were washed extensively with lysis buffer (0.3 M immunoprecipitation as described above with the indicated antibodies. relative to the total events.

determined by calculating the ratio of the gated BrdUrd-positive cells relative to the total events.

Going DNA replication, BrdUrd incorporation was identified using anti-

room temperature for 30 min. DNA content was analyzed, based on

histone H1. Reactions were terminated by adding 4

/MgCl2, 5 mM MnCl2, and 1 mM dithiothreitol). For autoactivation,
immobilized Nek9 or associated complexes were incubated in kinase buffer supplemented with 100 μM ATP at 25 °C for 20 min. Activation was stopped by either adding electrophoresis sample buffer, where appropriate, or by washing the beads in kinase buffer. Subsequently, activity was assayed by incubation at 30 °C in kinase buffer plus 5 μM ATP, 100 μCi/ml [γ-32P]ATP (Amersham Biosciences), and 1 ng of histone H1. Reactions were terminated by adding 4 × Laemmli SDS sample buffer and boiling. Denatured samples were resolved by SDS-PAGE.

Peptide Identification by Mass Spectrometry and Bioinformatics Analysis—The gel piece containing selected polypeptides was first reduced and pyridylethylated as described previously (21). Up to 0.2 μg of the enzyme trypsin (Promega) was added to the dried gel. After overnight incubation, the supernatant was removed, and the gel was extracted twice with adequate amounts of 0.1% formic acid. The supernatant and the extracts were combined, dried in a Speed-Vac, and resuspended in 0.1% formic acid immediately before use. Electrospray mass spectrometry was performed using a Finnigan Mat LCQ ion trap mass spectrometer interfaced with an ABI 140D high pressure liquid chromatograph (PerkinElmer Life Sciences). A 150 × 0.5-mm PE BEH (PerkinElmer Life Sciences) (5-μm particle diameter, 300-Å pore size) with mobile phases of A (0.1% formic acid in water) and B (0.05% formic acid in acetonitrile) were used. Peptides were eluted using the acetonitrile gradient and analyzed by "tri-

play" experiment as described (21). Data interpretation as well as correlation between the spectra and amino acid sequences within a human EST database were done by the SEQUEST Browser software package from Finnigan Corp.

RESULTS

Identification of Nek9 as a Novel Associated Protein of FACT—To identify key binding partners for the hSpt16-SSRP1 complex, we undertook the immunoaffinity purification scheme to isolate SSRP1-containing protein complexes from the cell nuclei. Nuclear extracts were subjected to immunoprecipitation with anti-SSRP1 monoclonal antibodies (clone 10D1). As shown in Fig. 1A, the major protein bands of 140 and 90 kDa correspond to hSpt16 and SSRP1, respectively, as confirmed by Western blot and mass spectrometric analyses (data not shown). Such affinity purification also recovers a 120-kDa polypeptide, which associates with the FACT heterodimer with a molar ratio of ~1–5. Subsequent spectrometric analysis of this band revealed a sequence match of the identified spectra to the coding sequence of the human Nek9 protein. To further characterize this protein, we generated and affinity-purified polyclonal antibodies against different parts of Nek9, termed αNek9-M (spanning amino acids 291–559) and αNek9-N (spanning amino acids 1–291). Both antibodies specifically recognize a 120-kDa polypeptide in the HeLa cell extracts as well as in the monoclonal antibody 10D1-targeted immunocomplex (Fig. 1B and data not shown). Other anti-SSRP1 monoclonal antibodies, whose approximate epitope regions have been mapped by using deletion constructs of SSRP1 (Fig. 1B, lower panel), were subjected to immunoprecipitation experiments as well. Nek9 was also detected in the immunoprecipitates pulled down by 7F8 but not in those targeted by 3E4 and 2B12, thus indi-
cating its specific association with distinct FACT complexes (Fig. 1B). Additionally, because 10D1 and 7F8 did not cross-react toward Nek9 immunohistochemically (Fig. 1, B and F, and data not shown), we excluded the possibility here that the co-immunoprecipitated Nek9 arose from nonspecific antibody-epitope binding. While performing a reciprocal co-immunoprecipitation experiment using the anti-Nek9 antibodies, we were able to identify FACT heterodimers in the αNek9-N immunocomplex (Fig. 1C). Immunoprecipitates targeted by αNek9-M, on the other hand, did not contain FACT (Fig. 4A and data not shown). Such observation suggests that αNek9-M targets a fraction of Nek9 that does not associate with FACT in the cells. Altogether, these immunoprecipitation results demonstrate the unique presence of a FACT/Nek9 protein complex in vivo.

Nek9 Interacts with FACT to Form an ~600-kDa Complex—To delineate unequivocally the specific interaction between Nek9 and FACT, we carried out an in vitro pull-down assay using baculovirus-derived recombinant proteins (Fig. 1D). Myc-tagged Nek9 was found to bind immobilized FLAG-FACT140 (lane 2) and His6-SSRP1 (lane 4), whereas no interaction between Myc-Nek9 and other similarly tagged proteins was observed (lanes 5 and 6). Interaction of Nek9 with SSRP1 was also verified through co-infection of respective recombinant baculoviruses followed by co-immunoprecipitation analyses using the M2 antibody (Fig. 1E).

Next, in a gel filtration chromatography (using Sephacryl S-400) of the HeLa nuclear extracts, Nek9 elutes with a molecular mass of ~600 kDa, which is consistent with previous observations (20) (Fig. 1F). FACT has overlapping but broader distributions in fractions and appears to exist in higher molecular size protein complexes. To further establish the co-elution of the FACT and Nek9 polypeptides, antibody 10D1 was used to carry out immunoprecipitation on selected fractions. Nek9 was found in the FACT-containing complexes isolated from fractions 20–28, coinciding with the elution profile of endogenous Nek9. Similar co-elution profile was also observed with the 7F8 antibody (data not shown). Taken together, these results validate the stable association of Nek9 with a distinct FACT complex in vivo, whose size is around 600 kDa. However, we cannot presently rule out the possibility of the presence of additional factors in the FACT-Nek9 complexes due to the larger-than-expected molecular mass for the heterotrimer.

Nek9 and FACT Partially Co-localize in the Interphase Nucleus and Are Similarly Free from Condensed Chromatin but Dissociate from Each Other during Chromatin Decondensation—The subcellular localization of the endogenous Nek9 protein was determined by indirect immunofluorescence by using the polyclonal antiserum directed against Nek9. Results of confocal microscopic analysis indicate that, consistent with the above biochemical evidence, Nek9 exhibits a nuclear distribution in HeLa cells (Fig. 2A, a and b). On the other hand, although ectopically expressed FLAG-Nek9 distributed predominantly in the nucleus (Fig. 2B, a), we also observed nucleocytoplasmic and cytoplasmic staining in some cells (Fig. 2B, b). To better characterize the nature of the endogenous FACT-Nek9 association, co-localization experiments were performed simultaneously. In the interphase nuclei, we detected a discernible degree of overlap in staining patterns between Nek9 and either component of the FACT complex (Fig. 2A, a and b). On the other hand, the presence of non-merged signals concurs with the above observation that a fraction of the endogenous Nek9 does not associate with FACT and vice versa. In cells that enter mitosis, the immunoreactive signals of Nek9, FACT140, and SSRP1 become similarly diffused and entirely excluded from the condensed chromatin (Fig. 2A, c–f; data not shown for FACT140). Interestingly, a significant dysjunction of anti-
FIG. 1. Identification of an endogenous FACT-Nek9 multiprotein complex. A, SSRP1-associated complexes were immunoprecipitated (IP) from HeLa nuclear extracts using SSRP1-specific monoclonal antibody 10D1, resolved by and shown on a Coomassie Blue-stained gel. Specific SSRP1-interacting protein bands that are absent in the control immunoprecipitates were identified by mass spectrometry. In addition to FACT subunits, an ~120-kDa protein was identified as the human Nek9 protein. B, Western blot (WB) analysis of the different immunoprecipitates targeted by control antibody 2B12, anti-SSRP1 mAbs, 3E4, 10D1, and 7F8, and HeLa cell extracts, as indicated. Immunoblotting was done using aFACT140 and aSSRP1 (10D1) mAbs (top panel) or aNek9-M antibody (bottom panel). The amount of the Input is equivalent to 1/40 of that of the immunoprecipitate (IP). The lower panel schematically represents the approximate regions of SSRP1 (in amino acids) in which the recognition sites of the different monoclonal antibodies may lie, as determined through immunoblotting on deletion constructs of SSRP1. C, Western blot analysis of the different immunoprecipitates targeted by aNek9-N (lane 1), pre-immune serum (PI, lane 2), and HeLa cell extracts (lane 3). Immunoblotting was done using aFACT140 and aSSRP1 (10D1) mAbs (lower panel) or aNek9-N antibody (top panel). The amount of the input is equivalent to 1/40 of that of the immunoprecipitate. D, M2 beads bound without (lane 1) or with (lane 2) FLAG-FACT140, or FLAG-p53 (lane 6) and nickel beads bound without (lane 3) or with (lane 4) His6-SSRP1 or His6-thioredoxin (lane 5) were used as baits in the in vitro pull-down assay. The presence and purity of the immobilized proteins were seen on Coomassie Blue-stained gels. Lysates from insect cells (Sf9) previously infected with Myc-Nek9-expressing baculoviruses were used in the binding reactions (lanes 1–4). Bound proteins were detected through immunoblotting with anti-Myc antibodies. All proteins, except for the bacterially expressed His6-thioredoxin, were derived from baculoviruses-infected insect cells. E, lysates of insect cells (Sf9) singly infected with His6-SSRP1-expressing baculoviruses (lane 1) or co-infected with FLAG-Nek9-expressing viruses (lane 2) were subjected to immunoprecipitation using the M2 beads (Sigma). Expressions of His6-SSRP1 (middle panel) and FLAG-Nek9 (lower panel) were monitored by immunoblotting using anti-His6 antibody (Qiagen) and M2 antibody, respectively. Components of the M2 immunoprecipitates, as shown by Coomassie Blue-stained gel, are as indicated. F, chromatographic fractions (numbers as indicated) generated by the gel filtration of HeLa nuclear extracts (using Sephacyl S-400) were immunoblotted with aFACT140 (clone 8D2), aSSRP1 (10D1), and aNek9-M antibodies. Equal amounts of selected fractions were further subjected to immunoprecipitation using mAb 10D1. Immunoprecipitates were also probed with aFACT140 (8D2), aSSRP1 (10D1), and aNek9-M antibodies.
Nek9 and anti-FACT staining patterns were observed in post-cytokinetic cells (Fig. 2A, g). FACT seems to reassociate with decondensing chromatin at this point, whereas Nek9 remains diffused and free from chromatin. However, in post-cytokinetic cells with less aggregated DAPI staining, representative of nucleus establishment and interphase initiation, the localization of Nek9 and FACT can be seen partially merged again (Fig. 2A, h). Together, these data demonstrate that the subcellular locations of Nek9, as well as FACT, alter throughout the cell cycle.

Upon Autoactivation, Nek9 Undergoes Phosphorylation-dependent Electrophoretic Mobility Shift and Phosphorylation on Thr210—Among Nek family proteins, there is extensive sequence conservation within the kinase domain, especially in the activation loop region. A previous study (19) using the peptide substrate demonstrated that Nek9 was able to phosphorylate in vitro the Thr210 residue within the activation loop, thus indicating the potential ability of Nek9 to autophosphorylate. To assess whether phosphorylation indeed occurs on well conserved Thr210 as well as the relationship between such phosphorylation and the kinase activity, we generated polyclonal antibodies that specifically recognize phosphorylated Thr210 of Nek9. Similar to the previously reported results on transfected FLAG-Nek9 (20), endogenous, immunoprecipitated Nek9 kinase became activated (i.e. phosphorylation on histone H1) upon pre-incubation with a high concentration of ATP (100 μM) (Fig. 3A, lane 3). Interestingly, we observed that the majority of Nek9 underwent electrophoretic mobility shift only at the high ATP concentration. Phosphorylation on Thr210 accompanies this decrease in mobility, as such a slower moving form was detectable using the phospho-specific antibody. The specificity of this antibody was revealed by the lack of immunoreactive signals from the non-shifted species of Nek9 (Fig. 3A, lanes 1 and 2). This observation also suggests that phosphorylation at Thr210 was absent or at very low levels on the non-activated Nek9 polypeptides. Note that the immunoprecipitates possessed minimal activity when pre-incubated with no ATP or low ATP, implying a likely requirement of endogenous Nek9 and its autoactivation for the optimal H1 kinase activity. The 10D1-targeted FACT-complexed Nek9 had similar enzymatic behaviors upon kinase activation (data not shown).

The ATP-induced electrophoretic retardation of Nek9 can be attributed to addition of the phosphate moiety, due to the fact that treatment with alkaline phosphatase following kinase autoactivation abolished such positional shift on the gel (Fig. 3B, lanes 1–3). Consistently, the accompanying immunoreactivity toward the Thr210 phospho-specific antibody was also found directly linked to phosphorylation. Previous reports (19, 20) have demonstrated that the mitotic form of Nek9 has a retarded electrophoretic mobility. To address the issue of whether the mitotic and the autoactivated, Thr210-phosphorylated form of Nek9 share similar characteristics, we performed immunoblotting on Nek9 isolated from different cell cycle phases. We could routinely observe differences in mobility among the in vitro autophosphorylated Nek9, mitotic Nek9, and Nek9 isolated from asynchronous culture (Fig. 3B, lanes 4–6). Furthermore, the mitotic Nek9 is not phosphorylated at the Thr210 residue (data not shown), additionally implying that these two forms of Nek9 may represent distinct species.

Phospho-Nek9 Associates with FACT in a Cell Cycle-dependent Manner—Next, to examine the possibility that the upshifted, Thr210-phosphorylated form of Nek9 is an endogenous entity, we probed for its presence in the cell extracts. Using the phospho-specific antibody, we were able to detect a protein band with the mobility and immunoreactivity characteristic of the activated form of Nek9 (Fig. 4A, lane 1). In addition, this
antibody also recognized a band, whose existence was phosphatase-sensitive (data not shown), in the 10D1-targeted (Fig. 4A, lane 3) and the T7S-targed (data not shown) immunocomplexes. Conversely, no phosphorylated form was found present in the αNek9-M immunoprecipitates, which are free of FACT complexes. These results strongly support the existence of an active, phosphorylated form of Nek9 as well as a phospho-Nek9-FACT complex in vivo. This specific and preferential association of the phosphorylated form of Nek9 with the FACT complex indicates that, in the presence of FACT, Nek9 may undergo a marked elevation in Thr210 phosphorylation.

In an attempt to further characterize the nature of the phospho-Nek9-FACT complex, we next inspected whether the level of Thr210 phosphorylation on FACT-bound Nek9 was regulated in a cell cycle-dependent manner. FACT complexes at different stages of the cell cycle were immunoprecipitated and subsequently probed for the Thr210-phosphorylated Nek9. As shown in Fig. 4B, there is a constitutive presence of phospho-Nek9 in the FACT immunocomplexes throughout different phases. However, the levels of association seemed to rise upon release from the G1/S phase block (lanes 1–4) and undergo detectable reduction in the M and G2 phases (compare lanes 5 and 6). Such observed variation potentially reflects an interphase-specific elevation in the formation of the active, phospho-Nek9-FACT complex.

RNA-mediated Knock-down of Nek9 Proteins Triggers Interphase Delay—Next, toward elucidating the cellular roles of Nek9 and further examining its link to interphase, we undertook a vector-based small interfering RNA approach to abrogate endogenous Nek9 expression in HeLa cells. Successful establishment of independent clones stably harboring Nek9-interfering dsRNA was confirmed by Western analysis on cell lysate proteins, as shown in Fig. 5A. While the level of control protein remained the same, <30% of the endogenous Nek9 polypeptides were detected in the two Nek9RNAi clones as compared with the control clones.

While maintaining the culture of these stable clones, we unexpectedly observed relatively slower growth of the Nek9RNAi cells. To examine this phenotype more closely, we performed colony formation assay. Independent experiments have demonstrated that, after 2-week culturing, Nek9RNAi cells yielded dramatically smaller colonies than the control cells, clearly a consequence of a stalled cell growth (Fig. 5B). To further explore the possible underlying attributes of such retarded proliferation, these stable cells were subjected to synchronization studies. First, cells were synchronized through nocodazole-mitotic shake-off treatment. At different time points, cells were collected, and DNA content was determined by flow cytometry. As shown in Fig. 6A, the rates of appearance for cells with a DNA content of 2N (representative of G1 phase) were unchanged regardless of the levels of Nek9 proteins. However, the Nek9RNAi cells seemed to move through the G1 phase at a slower pace and consequently initiate S phase ~3–4 h later than control cells.

In a separate experiment, double thymidine block was used to monitor cell cycle progression through the G1/S point. Results depicted in Fig. 6B evidently indicate a delayed S phase in the Nek9RNAi culture. On the other hand, the progression of G2/M phase appeared normal in this cell line, comparable with the above nocodazole experiment. A similar experiment was
Fig. 4. Phospho-Nek9 associates with FACT in a cell cycle-dependent manner. A, HeLa cells were immunoprecipitated (IP) with the indicated antibodies: control antibody (lane 2), αSSRP1 mAb 10D1 (lane 3), rabbit pre-immune (PI) sera (lane 4), and αNek9-M antibody (lane 5) (cell extract input is shown in lane 1). The presence of Nek9, FACT heterodimers, and phospho-Nek9 was detected by Western blot (WB) analysis using αNek9-M antibody (top), αFACT140 and αSSRP1 antibodies (middle), and α-phospho-Thr210 antibody (bottom), respectively. Position of the upshifted, phosphorylated Nek9 is indicated by the upper arrow, and the lower arrow points to the presumed usual location of Nek9. B, FACT-associated immunocomplex was isolated by αSSRP1 mAb 10D1 from HeLa cells at different cell cycle stages: exponentially growing (lanes 1 and 5), G1/S phase (lane 2), S phase at 1 h after double-thymidine block (lane 3), S phase at 3 h (lane 4), M phase (lane 6), and G2 (lane 7). The presence of Nek9, FACT heterodimers, and phospho-Nek9 was detected by αNek9-M antibody (top), αFACT140 and αSSRP1 antibodies (bottom), and α-phospho-Thr210 antibody (middle), respectively. The arrowhead designates the location of the Thr210-phosphorylated form of Nek9. The graph at the bottom illustrates the changes in the degree of Thr210 phosphorylation (on FACT-associated Nek9) across different phases. The degree of phosphorylation, expressed as a ratio in band intensity of phospho-Nek9 to total Nek9, was normalized to the asynchronous group. A5, autoactivated species; M, M phase.

also carried out to quantitatively assess the population of actively replicating cells at different times upon removal of the block. Cells were pulse-labeled with BrdUTP before being harvested at the designated time points. The ratio of the BrdUrd-positive cells, termed the “labeling index,” represents the population of cells that actively synthesize DNA in a particular culture. By plotting the changes in the labeling indexes in relation to time, a similar trend was observed in which the Nek9RNAi cells exhibited markedly delayed S phase, specifically at the onset stage (Fig. 6C). Collectively, data from the RNAi experiments indicate that an adequate level of Nek9 is essential for G1/S transition and S phase progression.

DISCUSSION

In the present study, we identified Nek9 as a novel component of the FACT-associated multiprotein complexes, and we performed functional characterization of this protein. Their in vivo interaction was demonstrated by using biochemical as well as cell biological methods. Most notably, a considerable portion of the endogenous Nek9 polypeptides are stably bound to FACT, although gel filtration and immunoprecipitation analyses indicated that the FACT-Nek9 complex represents only a fraction of the total FACT-associated complex populations. It is interesting to note that the only presently known human FACT-interacting proteins are both kinases. Besides Nek9, CK2 was recently found to form an UV-induced, p53-targeting complex with FACT (22, 23). Although both complexes differ physically and functionally, their existence consistently implies FACT in the progression of the cell cycle (i.e., interphase) and further supports a potential role of post-translational modification (i.e., phosphorylation) in FACT-associated processes.

Our immunoprecipitation experiments using different αSSRP1 and αNek9 antibodies have provided a qualitative assessment of the interaction between endogenous FACT and Nek9. Due to the dissimilar targeting epitopes, different clones of antibodies raised against a single protein may pull down distinct sub-complexes of that target protein (or no protein at all, as in the case of our 2B12 antibody). “Masking” of the epitopes, as a consequence of conformational changes or interacting proteins binding, may cause this. It is also possible that antibody-epitope binding may lead to a disruption of protein...
interaction. Such characteristics of antibody thus explain the inability of 3E4 and 
H9251 Nek9-M to respectively co-immunoprecipitate Nek9 and FACT and, in turn, justify the presence of 
FACT-free Nek9 complexes (and vice versa) in the cells. This 
observation that only a fraction of FACT associates with Nek9 
(and vice versa) was further strengthened by results of the gel 
filtration and immunostaining analyses (Figs. 1 
and 2).

Despite the presence of a nuclear localization signal in Nek9, 
previous reports (19, 20) have demonstrated a cytoplasmic 
localization of this protein as well as its putative roles related 
to its subcellular location (i.e. regulation of microtubule 
dynamics and mitosis). In this report, we have provided several lines 
of experimental evidence supporting the existence of a nuclear 
counterpart of the human Nek9 protein as follows: 1) the immunochemical detection of Nek9 in the nuclear extract prepa-
rations; 2) the biochemical demonstration of the stable association 
of a large pool of Nek9 polypeptides with the FACT 
heterodimers, which are primarily nuclear factors; and 3) the 
imunocytochemical characterization of Nek9’s nuclear local-
ization and association with euchromatin (acetyl-histone H3) 
regions (data not shown).

Our results uncovered a novel functional aspect of Nek9 that 
is different from previous reports, the involvement in the 
progression of interphase. By using Nek9-specific antibody mi-
croinjection in prophase cells, Roig et al. (20) have observed 
spindle and chromosome abnormalities characteristic of mis-
regulated mitosis. However, when antibodies were injected in 
interphase cells, they failed to enter mitosis, indicative of 
stalled cell cycle progression. Such preliminary observation is 
in agreement with the behaviors of our Nek9RNAi cells, further 
pinpointing an equally important role of Nek9 in the inter-
phase. On the other hand, based on our synchronization/DNA 
content analysis on the knock-down cell line, no delay or ab-
normal progression of mitosis was detected. It is possible that

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**Fig. 6.** Slower growth of Nek9RNAi cells can be partly attributed to prolonged G, and S phases. A, cells stably harboring control or Nek9-targeting dsRNA were synchronized at G/M as described under “Experimental Procedures.” Cell cycle progression was analyzed based on DNA content by FACS. B, control and Nek9RNAi cells were synchronized at the G/S junction by double thymidine block. At the indicated time points after the release, cells were harvested and subjected to DNA content analysis by FACS. C, control and Nek9RNAi cells were synchronized at the G/S junction and pulse-labeled with BrdUTP before being harvested at different time points as in B. Cell cycle profiles and degree of BrdUrd labeling (i.e. active DNA replication) were monitored by FACS. The labeling index quantitatively represents the percentage (%) of BrdUrd-positive cells in a counted pool of cells.
the mitotic activity of Nek9 would not be compromised unless the protein levels are further diminished below a critical threshold. Interestingly, results obtained from our cell cycle analysis of the Nek9RNAi cells bear close resemblance to previous mutant phenotypic studies in the yeast Saccharomyces cerevisiae. CDC68, the yeast counterpart of the human SPT16 gene, was conditionally arrested in the G1 stage (10). In an independent study, mutation in POB3, the homologue of SSRP1, was found to cause delay in S phase onset and progression (11). Correlation among these cell cycle-related mutant phenotypes directly strengthens the physiological relevance of the FACT-Nek9 interaction. Furthermore, the observed role in interphase may be temporally related to an increase in Thr210 phosphorylation on the FACT-bound Nek9 (Fig. 4B). Based on these results, we postulate that Nek9, in association with FACT, may be actively involved in certain important FACT-related functions, compromise of which would ultimately lead to impeded interphase progression.

Results of the co-localization experiments revealed an interesting feature of the formation of the endogenous FACT-Nek9 complexes; although normally existing as an otherwise stable complex, FACT-Nek9 briefly separates at the M-G1 transition. We performed additional immunoprecipitation experiments with lysates derived from cells arrested at different cell cycle phases, and we found no differences in the co-immunocomplex profiles as compared with that of the asynchronous culture (Fig. 4B and data not shown). However, it may be difficult to detect such absence of complex association due to its transient nature as well as depending on the lysate extraction condition. Nevertheless, our observation of the divergent localization between FACT and Nek9 at the end of mitosis signals a physical, and possibly functional, separation of the FACT-Nek9 complex at this particular stage. It is also possible that the formation of such a protein complex has a temporal dependence on the establishment of nuclear envelope and appropriate chromatin state. Furthermore, the novel observation of the cyclic manner in which FACT becomes excluded from condensed chromatin and then reassociates with the decondensing one is consistent with the putative mode of action of FACT, facilitating the accessibility of chromatin. Such regulated movement can prevent any untimely loosening of the condensed chromosomes, which is catastrophic to the mitotic progression.

Our results on the mechanistic characterization of Nek9 provide another example of enzymatic autoactivation. Although it is currently unclear why the higher-than-physiological concentration of ATP is required for activation, some of the above characteristics are reminiscent of some other kinases such as Aurora A kinase and Polo-like kinase Plk-1 (24–26). Consistent with the previous observations, our results based on the use of the anti-phospho-Thr210 antibody have demonstrated an important role of the activation loop structure in the upregulation of kinase. Based on the known structural model for kinase domains, it is probable that phosphorylation at the threonine location may lead to negative charge-mediated structural changes (i.e., catalytic pocket opening), which ultimately are more favorable for substrate targeting.

Most notably, utilizing the phospho-specific antibody, we have achieved the novel finding on the existence of the lower mobility, Thr210-phosphorylated species of Nek9 in vivo. Due to the extensive kinase domain conservation among the Nek family members, it is possible that catalytic attributes of Nek in vitro and in vivo are widely shared by others. The presence of the phosphorylated Nek9 in the FACT-associated immunocomplex indicates that a fraction of the complexed Nek9 remains in a steady-state active form. The finding of the preferential association of phospho-Nek9 with FACT in vivo provides evidence that interacting with FACT may render the kinase more active. The possibility of FACT acting as a non-kinase activator of Nek9 as well as the exact mechanism through which FACT alters the catalytic property of Nek9 awaits further study.

The complex and multiple roles of FACT heterodimer in DNA replication, transcription initiation, and transcription elongation are well established through various genetic and biochemical studies done on different homologs. Based on the results obtained from the yeast system, Formosa et al. (9) postulated that this heterodimer has a single central activity of altering the chromatin, although its roles at different stages of replication and transcription might be mediated through coordinately functioning with other factors. This interaction network has been experimentally established by the discovery of various genetic and physical interactors of FACT. It is interesting to note that, upon gel filtration on crude extract materials (Fig. 1F), the peak fractions of FACT were in a range that is much higher than the expected native mass of purified FACT, which is about 230 kDa (1). Such a faster and broad elution profile of FACT provides additional strong evidence for the existence of different multiprotein complexes. Surprisingly, in the immunoprecipitates of some of the fractions, our FACT-specific antibodies could detect additional protein bands that had different electrophoretic mobilities (Fig. 1F). This raises the possibility that, on top of protein interaction, post-translational modifications may also be present and of some significance in the regulation of FACT activities. Finally, the addition of Nek9 to the growing list of FACT-interacting factors may either suggest a novel pathway of this FACT complex or indicate the involvement of phosphorylation-dependent signal transduction in the existing FACT-associated processes. Thus, identification of physiological substrates targeted by the kinase complex may be of great importance in its functional characterization.

Acknowledgments—We are grateful for the bioinformatic work performed by Dr. Chun-Hua Hsu. We especially thank Dr. Stephen J. Lippard for generously providing the SSRP1 expression vector. Drs. Danny Reinberg and Ruey-Hwa Chen for critical discussions, and Dr. You-Yun Tsay for assistance with mass spectrometric analysis.

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