Substrate Specificity and Cell Cycle Regulation of the Nek2 Protein Kinase, a Potential Human Homolog of the Mitotic Regulator NIMA of Aspergillus nidulans

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The human Nek2 protein kinase is the closest known mammalian relative of the mitotic regulator NIMA of Aspergillus nidulans. The two kinases share 47% sequence identity over their catalytic domains and display a similar cell cycle-dependent expression peak at the G2 to M phase transition. Hence, it is attractive to speculate that human Nek2 and fungal NIMA may carry out similar functions at the onset of mitosis. To study the biochemical properties and substrate specificity of human Nek2 and compare them to those reported previously for other NIMA-related protein kinases, we have expressed Nek2 in insect cells. We show that recombinant Nek2 is active as a serine/threonine-specific protein kinase and may undergo autophosphorylation. Both human Nek2 and fungal NIMA phosphorylate a similar, albeit not identical, set of proteins and synthetic peptides, and ß-casein was found to be a suitable substrate for assaying Nek2 in vitro. By exploiting these findings, we have studied the cell cycle regulation of Nek2 activity in HeLa cells. We show that Nek2 activity parallels its abundance, being low during M and G1, but high during S and G2 phase. Taken together, our results suggest that human Nek2 resembles fungal NIMA in its primary structure, cell cycle regulation of expression, and substrate specificity, but that Nek2 may function earlier in the cell cycle than NIMA.

In the filamentous fungus, Aspergillus nidulans, entry into mitosis requires the activation of a serine/threonine protein kinase termed NIMA (1, 2) (reviewed in Ref. 3). Cells carrying a temperature-sensitive copy of the nima gene arrest specifically in G2 when shifted to the restrictive temperature (hence nima for never in mitosis), and they enter mitosis synchronously when released from the block (4, 5). Conversely, strong overexpression of a wild-type nima allele drives cells into a premature mitotic state from any point in the cell cycle, the phenotype of such cells being characterized by the formation of mitotic spindles and the maintenance of chromosomes in a condensed state (1, 6, 7). Expression of catalytically inactive NIMA or the carboxyl-terminal noncatalytic domain of NIMA also causes a G2 arrest, even in cells expressing wild-type NIMA, indicating that these NIMA mutants act in a dominant-negative fashion (7). How exactly NIMA interacts or cooperates with the p34cdc2/cyclin B protein kinase to trigger entry into mitosis in A. nidulans is an interesting but still unresolved question (2).

The striking evolutionary conservation of cell cycle control mechanisms (8) suggests that protein kinases related to fungal NIMA may also exist in higher eukaryotes. In support of this possibility, three NIMA-related protein kinases, termed Nek1, Nek2, and Nek3 (for NIMA-related kinase), have recently been cloned from mammalian species. Nek1 was identified by screening of a mouse cDNA expression library with anti-phosphotyrosine antibodies, and this kinase was shown to display dual specificity for tyrosine and serine/threonine residues (9, 10). Nek1 may play a role specifically during meiosis, since high levels of nek1 mRNA were expressed in male and female germ cells (10). Independently, three cDNAs coding for human NIMA-related kinases were isolated by using a polymerase chain reaction approach (11). One cDNA was closely related to murine nek1, but the two others represented novel NIMA-related kinases and hence were named Nek2 and Nek3 (12).

Among all vertebrate kinases known to date, Nek2 is most closely related to fungal NIMA; furthermore, the two kinases display similar cell cycle-dependent expression patterns, being low in G1 and increasing through S and G2 to reach a plateau in late G2/M, suggesting that they may both play a role at the onset of mitosis (12, 13). Unfortunately, further progress toward understanding Nek2 function had been hampered by the lack of biochemical information on Nek2. In particular, it had previously not been possible to measure specific Nek2-associated kinase activity in Nek2 immunoprecipitates prepared from mammalian cells, and it could not be determined whether these negative results were due to the low abundance of Nek2 in exponentially growing cells, a narrow window of activity during the cell cycle, the presence of inhibitory factors in whole cell lysates, the choice of inappropriate substrates or assay conditions, or a combination of these parameters (12).

To overcome this major difficulty and make Nek2 amenable to biochemical analyses, we have now generated recombinant baculoviruses and expressed wild-type human Nek2, as well as a catalytically inactive mutant, in insect cells. We show here that recombinant human Nek2 is indeed active as a serine/threonine-specific protein kinase. Furthermore, we report that

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1 The abbreviations used are: Nek, NIMA-related kinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter; MAP, microtubule-associated protein; PLM, phospholemna-derived peptide.
the biochemical properties and substrate specificity of human Nek2 are remarkably similar to those of fungal NIMA; in contrast, Nek2 can readily be distinguished from Nek1, as well as from casein kinase II, a ubiquitously expressed enzyme which also prefers casein as its in vitro substrate. Finally, having optimized the conditions for assaying Nek2 activity, we have been able to return to cultured HeLa cells and demonstrate endogenous Nek2 kinase activity. We found that Nek2 activity fluctuates during the cell cycle, strengthening the hypothesis that this NIMA-related human kinase protein may play a role in cell cycle regulation.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculovirus—To create a baculovirus encoding wild-type human Nek2 (pBlueBacNek2), a full-length human Nek2 cDNA was excised as a NasiI-PstI fragment from pGEM-Nek2 (12) and subcloned into the BamHI (blunted)-PstI site of the baculovirus transfer vector pBlueBac (Invitrogen Corp.). In this construct the Nek2 cDNA is placed behind the viral polyhedrin promoter, but the authentic ATG is used for translational initiation. Nek2 recombinant baculovirus was then generated by in vivo homologous recombination following cotransfection of pBlueBac-Nek2 and wild-type baculoviral DNA into Sf9 cells. The transfection supernatant was used to infect Sf9 cells in a standard plaque assay, recombinant virus was purified by three further rounds of plaque assays, and purity was confirmed by the absence of polyhedrin protein bands. All procedures relating to Sf9 insect cell growth, transfections, infections, plaque assays, and viral amplification were as previously described (14).

A catalytically inactive mutant of Nek2 (Nek2-K37R, carrying a replacement of Lys-37 by Arg) was created by site-directed mutagenesis of pGEM-Nek2, using the Clontech mutagenesis kit, and the mutation was confirmed by double-stranded plasmid sequencing. The mutagenic oligonucleotide used was AGATTTATTGGTTAGAGAACAGTGCTATGGC, with the underlined codon corresponding to residue 37 in Nek2; as a selection primer, an oligonucleotide eliminating the PstI site in the pGEM mutagenic site was used. The full-length mutant cDNA was excised from the plasmid pGEM-Nek2-K37R and subcloned into the PstI (blunted)-XbaI site of the vector pVL1392 (Pharmingen Corp.) to create pVL1392-Nek2-K37R. Recombinant Nek2-K37R baculovirus was generated by cotransfection of pVL1392-Nek2-K37R and BaculoGold DNA (Pharmingen Corp.) and subsequent amplification of viral DNA.

Protein Expression, Preparation of Cell Extracts, and Immunoprecipitation—Sf9 cells were infected with recombinant baculoviruses in 10-cm culture plates at a multiplicity of infection of 10. Cell lysates were prepared at 48 h post-infection (unless indicated otherwise) after harvesting using 1X PBS, 1% Triton X-100, and protease inhibitors. Cells were lysed by sonication followed by centrifugation at 13,000 ×g for 10 minutes at 4°C. The supernatant was transferred into new tubes and washed twice with ice-cold PBS, 1 mM phenylmethylsulfonyl fluoride, and resuspended in 100 μl lysis buffer (50 mM Hepes/KOH, pH 7.5, 100 mM NaCl, 5 mM KCl, 10 mM MgCl2, 2 mM EDTA, 5 mM EGTA, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 0.3 mM sodium vanadate, 20 μM β-glycerophosphate, 20 mM sodium fluoride). After 30 min on ice, cells were lysed by Dounce homogenization, and lysis was checked by phase contrast microscopy. Lysates were spun at full speed in a microcentrifuge, and the supernatants are referred to as whole cell extracts. We found that Nek2 kinase was inhibited by several detergents, notably those commonly used in cell extraction buffers. Nonidet P40 (1%), Triton X-100 (1%), or SDS (0.1%) all strongly inhibited the activity of immunoprecipitated Nek2 (data not shown). However, it should be noted that extracts were prepared under appropriate conditions (see above) and stored at −80 °C, Nek2 kinase activity in whole cell extracts or immunoprecipitated extracts was stable for several months.

Prior to immunoprecipitation, extracts were equilibrated for protein content where appropriate, and preclared with protein A-Sepharose beads for 30 min. Extracts were then incubated for 60 min on ice with 0.01 volume of R3anti-Nek2 polyclonal rabbit antiserum (12), followed by protein A-Sepharose for a further 45 min. Immune complexes were collected by centrifugation and washed three times with 1 ml of lysis buffer before they were used for in vitro kinase assays. Alternatively, they were stored at −80 °C or mixed with an equal volume of 3 × gel sample buffer and analyzed by SDS-PAGE.

In Vitro Kinase Assays—During the initial studies, immunoprecipitated recombinant Nek2 was assayed at 2000 × 106 cpm/gel at 4°C, and then reactions were carried out for 20 min at 30 °C in Nek2 kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2). Then, kinase reactions were carried out for 20 min at 30 °C in Nek2 kinase buffer supplemented with 4 μM ATP, 1 mM dithiothreitol, and 10 μCi of [γ-32P]ATP (Amersham Corp.), in a total volume of 50 μl. Dephosphorylated casein (Sigma, C-4032) was included at 0.5 mg/ml as an exogenous substrate, unless indicated otherwise. Reactions were stopped by the addition of 50 μl of 3 × gel sample buffer and heating to 95 °C. Reactions products were then analyzed by SDS-polyacrylamide gel electrophoresis. Casein kinase I (Promega Corp.; 2.5 units/assay) was assayed under the same conditions except that 200 μM NaCl was added to the kinase buffer.

Following optimization of assay conditions, the activity of endogenous human Nek2 kinase was detected as follows. HeLa cells were lysed in 50 mM Hepes, pH 7.5, 5 mM MnCl2, 10 mM MgCl2, 5 mM EGTA, 2 mM EDTA, 100 mM NaCl, 5 mM KCl, 0.1% Nonidet P-40, 30 μg/ml of DNase I, 30 μg/ml of RNase A, 1 μM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin and pepstatin A, 0.1% aprotinin, 1 μM okaidic acid, and 1 μg/ml heparin. Then, Nek2 was immunoprecipitated as described above. Immunoprecipitates were then given three final washes in lysis buffer, and kinase assays were carried out as described above, except for using the following, modified assay buffer: 50 mM Hepes, pH 7.5, 5 mM MnCl2, 5 mM NaF, 5 mM β-glycerophosphate, 1 μM okaidic acid, and 1 μg/ml heparin.

Peptide phosphorylation was assayed by both phosphocellulose P81 paper binding (15) and by thin layer chromatography in buffer C (isobu- tyric acid/pyridine-acetic acid butanol:water = 65:5:3:2:29), essentially as described previously (16); 500 μM peptide was used in each assay. Metabolic Labeling, Phosphoamino Acid Analysis, and Phosphopeptide Mapping—Metabolic labeling of baculovirus-infected Sf9 cells was carried out as follows. 24-h post-infection cultures were washed twice in either methionine- or phosphate-free medium, and then replated, respectively, in methionine-free medium containing 50 μM[35S]methionine or phosphate-free medium containing 500 μM[32P]orthophosphate; both labeling media also contained 10% dialyzed serum and 10% normal, fully supplemented Sf9 medium. Labeling was allowed to proceed for 16 h before preparation of cell extracts and immunoprecipitation of recombinant proteins.

Phosphoamino acid analyses and tryptic phosphopeptide mapping experiments were carried out as described elsewhere (16), except that the electrophoresis of tryptic peptides was carried out in 1.9 buffer (glacial acetic acid/formic acid (100:2) : deionized water, 156:44:100) for 3 h at 4°C.

Production of Nek2-specific Monoclonal Antibodies—A histidine- tagged fusion protein encoded by a partial nck2 cDNA (HePK 21) (11) was expressed in E. coli and purified by nickel column chromatography, as previously described (12). This fusion protein was then injected subcutaneously into Balb/c mice (about 50 μg/injection), and hybridoma cell lines were generated as described previously, using a direct hybridoma cloning method (18). The monoclonal antibody used in this study, termed NKL1, is an IgG1.

Immunoblotting—Proteins were resolved on 12% SDS-polyacryl amide gels and transferred to nitrocellulose by semi-dry blotting. Detection of Nek2 protein was then carried out using subcutaneously into Balb/c mice (about 50 μg/injection), and hybridoma cell lines were generated as described previously, using a direct hybridoma cloning method (18). The monoclonal antibody used in this study, termed NKL1, is an IgG1.

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Cell Cycle Synchronization—HeLa cells were synchronized at the G1/S boundary by the addition of 2 μg/ml aphidicolin (Sigma) for 16 h. For synchronization at prometaphase, cells were incubated for 16 h with 500 ng/mg nocodazole (Janssen), and mitotically arrested cells were collected by gentle pipetting. Cells were released from these blocks by three washes in PBS and replating into fresh medium. The cell cycle distribution of various HeLa cell populations was monitored by flow cytometry (FACS) analysis. For the purpose of these experiments, 106 cells were washed twice in ice-cold PBS and then suspended in 0.3 ml of PBS. Then, samples were fixed by slow addition of 0.9 ml of 95% ethanol (−20 °C) while vortexing. At this stage, cells were stored for up to 24 h. Immediately prior to FACS analysis, fixed cells were gently washed three times in ice-cold PBS, and then resuspended in 0.5 ml of 0.5% PBS. Then, 20 μl of RNase A (Sigma, 10 mg/ml, pre-boiled) and 25 μl of propidium iodide (200 μg/ml in 50 mM sodium citrate, pH 7.6) were added.
FIG. 1. Casein kinase activity of baculovirus-expressed Nek2 protein. Insect Sf9 cells were infected with the Nek2-recombinant baculovirus (Nek2), and whole cell extracts were prepared 36 h post-infection; for control (C), uninfected cells were analyzed in parallel. A, crude extracts were incubated with [γ-32P]ATP in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of casein; then, phosphoproteins were separated by SDS-PAGE and detected by autoradiography. Note that viral infection caused an overall decrease in protein expression. Molecular masses (kDa) are shown on the left, and the positions of the Nek2 protein and β-casein are indicated on the right. B, Nek2 was immunoprecipitated from insect cell extracts using either the Nek2-specific R31 polyclonal immune serum (I) or the preimmune serum (P). Immunoprecipitates (lanes 5–8) were incubated in the presence of [γ-32P]ATP and casein before SDS-PAGE and autoradiography. To confirm the specificity of the antibody and the size of Nek2, immunoprecipitates were prepared in parallel from in vivo [35S]methionine-labeled Sf9 cells (lanes 1–4) and also analyzed by SDS-PAGE and autoradiography. Molecular masses (kDa) are shown on the left, as the positions of the Nek2 protein and β-casein are indicated on the right.

Samples were incubated on ice for 30 min before the DNA contents were determined by measuring fluorescence intensities on a FACS II (Becton Dickinson) instrument.

RESULTS

Expression of Active Human Nek2 Protein Kinase in Insect Cells—The predicted amino acid sequence of Nek2 displays all the hallmarks of a protein kinase (20). Yet, no direct biochemical evidence for Nek2 kinase activity had previously been obtained. To be able to characterize this novel protein kinase, we expressed it from a recombinant baculovirus in insect Sf9 cells. Sf9 cells were infected with either a wild-type baculovirus or a recombinant Nek2 virus, and whole cell lysates were prepared at increasing times after infection. As determined by immunoblotting, expression of the 46-kDa Nek2 protein could first be detected at 18–24 h post-infection and reached maximal levels by 36–48 h (data not shown). At these latter times, a second, slower migrating form of Nek2 became visible, suggesting that Nek2 may be subject to post-translational modification in insect cells.

To detect Nek2-associated kinase activity, in vitro kinase assays were carried out on either whole insect cell lysates (Fig. 1A) or Nek2 immunoprecipitates (Fig. 1B). Where indicated, dephosphorylated casein was added as an exogenous substrate, since β-casein had previously been reported to be a preferred substrate of A. nidulans NIMA (13, 21). Whereas no major specific substrates of Nek2 could be detected among total insect cell proteins (Fig. 1A, compare lanes 1 and 2), β-casein was strongly phosphorylated in lysates from Nek2 virus infected insect cells (Fig. 1A, lane 4), but not in lysates from uninfected cells (Fig. 1A, lane 3). Similarly, strong β-casein phosphorylation was observed in Nek2 immunoprecipitates prepared from Nek2 virus infected cells (Fig. 1B, lane 8), but not in immunoprecipitates prepared from noninfected cells (Fig. 1B, lane 6) or in control immunoprecipitates prepared with preimmune serum (Fig. 1B, lanes 5 and 7). In Nek2 immunoprecipitates from Nek2 virus infected cells, a second protein migrating at approximately 46 kDa was strongly phosphorylated (Fig. 1B, lane 8). As indicated by comparison with immunoprecipitates prepared in parallel from [35S]methionine labeled cell lysates (Fig. 1B, lanes 1–4), this protein corresponded exactly with the recombinant human Nek2 (Fig. 1B, lane 4), suggesting that Nek2 protein kinase might undergo autophosphorylation (see also Fig. 1A).

The above results strongly suggested that the recombinant Nek2 protein displayed intrinsic kinase activity, but it remained formally possible that insect protein kinases might contaminate Nek2 immunoprecipitates. Thus, we constructed a recombinant baculovirus expressing a mutant Nek2 protein, termed Nek2-K37R, in which a critical lysine of the Nek2 catalytic domain (residue 37) was changed to arginine; by analogy to corresponding mutations made previously in other protein kinases including NIMA (21), this mutation was expected to render Nek2 catalytically inactive (22). As shown in Fig. 2A, the Nek2-K37R mutant was expressed to a similar level as the wild-type Nek2, and it was also immunoprecipitated with comparable efficiency (not shown). Interestingly, however, the Nek2-K37R protein did not display the retarded electrophoretic phosphorylation seen with the wild-type protein (Fig. 2A, lane 2 vs. lane 4). These results strongly suggest that the recombinant Nek2 protein displays intrinsic kinase activity.

FIG. 2. Kinase activity of wild-type but not K37R mutant Nek2. A, detection of Nek2-K37R (lane 1) or wild-type Nek2 (lane 2) protein in whole cell extracts prepared from insect Sf9 cells infected with the corresponding recombinant baculoviruses. Immunoblots were carried out with the R31 anti-Nek2 antibody. Molecular masses (kDa) are shown on the left. Immunoreactive bands migrating below 46 kDa represent degradation products arising during late viral infection. B and C, casein kinase activity associated with wild-type Nek2 (upper panel in B; dots in C) and Nek2-K37R mutant protein (lower panel in B; triangles in C). Kinase assays were carried out with immunoprecipitates prepared from baculovirus infected Sf9 cells. These were incubated at 30 °C, and reactions were stopped at the times indicated (minutes). Samples were separated on SDS-PAGE, then the gels were dried and autoradiographed. The positions of α- and β-casein are indicated. For Panel C, the β-casein band was cut out of the dried gels, and the radioactivity (cpm) associated with each band was determined by scintillation counting.
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Fig. 3. Expression of Nek2 in Sf9 cells does not cause an increase in tyrosine phosphorylation levels. Sf9 insect cell lysates were prepared 48 h post-infection with no virus (lane 1, C), wild-type Nek2 virus (lane 2, wt), mutant Nek2-K37R virus (lane 3, K37R), or p60src-encoding virus (lane 4, src). Equal amounts of protein from each lysate were then processed by SDS-PAGE and probed by immunoblotting with the anti-phosphotyrosine antibody, 4C10. Molecular masses are indicated on the left, and the position of the tyrosine phosphorylated p60src protein is marked on the right.

mobility that was characteristically observed with wild-type Nek2, indicating that the inactive kinase was not post-translationally modified to the same extent (Fig. 2A, compare lanes 1 and 2). Also, whereas the extent of β-casein phosphorylation catalyzed by wild-type Nek2 increased linearly with reaction time, the Nek2-K37R mutant was completely inactive in an in vitro kinase assay (Fig. 2B and C).

Biochemical Characterization of Recombinant Nek2 Protein Kinase—Prompted by the description of Nek1 as a dual-specificity kinase (10), we next asked whether human Nek2 might display any tyrosine kinase activity. To this end, Sf9 cells were infected with viruses encoding either wild-type Nek2 or the Nek2-K37R mutant, and extracts were probed by immunoblotting with an anti-phosphotyrosine antibody (Fig. 3, lanes 2 and 3). To provide negative and positive controls, lysates from uninfected Sf9 cells and from cells infected with a virus encoding the tyrosine kinase p60src were analyzed in parallel (Fig. 3, lanes 1 and 4). Only lysates from cells expressing p60src showed an increase in the overall level of phosphotyrosine, with p60src itself being the most prominent tyrosine-phosphorylated protein visible (Fig. 3, arrow). In contrast, no specific signals were produced by anti-phosphotyrosine antibodies on immunoblots of either lysates of Nek2 virus infected cells (Fig. 3, compare lane 2 with lanes 1 and 3) or Nek2 immunoprecipitates (data not shown). These data argue that Nek2 is likely to be a serine/threonine-specific kinase, like Aspergillus NIMA but unlike the murine dual-specificity kinase Nek1. This conclusion is supported also by the results of phosphoamino acid analyses performed on several 32P-labeled substrates of Nek2 (see below).

As summarized in Table I, the casein kinase activity of human Nek2 displays several characteristics that are reminiscent of the casein-kinase activity described for A. nidulans NIMA, but can readily be distinguished from that of another major casein kinase, casein kinase II (for review see Ref. 23). First, like NIMA but unlike casein kinase II, Nek2 kinase was not able to utilize [γ32P]ATP as a phosphate donor, and phosphate transfer from [γ32P]ATP could not be inhibited by the addition of unlabeled GTP up to concentrations of 500 μM. Second, Nek2 casein kinase activity was unaffected by heparin (up to 5 μg/ml), whereas substantial inhibition of casein kinase II occurred at the same concentrations, as expected (23). In further studies, we found that the β-casein kinase activity of Nek2 was sensitive to increases in ionic strength, with 200 mM NaCl leading to an approximate 50% (reversible) reduction in activity. Importantly, Nek2 activity was stimulated about 5-fold by 10 mM Mn2+, unaffected by 10 mM Ca2+, and completely inhibited by 10 mM Zn2+. Variation of the Mn2+ concentration (from 0 to 50 mM), in the presence or absence of 10 mM Mg2+, revealed that maximal stimulation of Nek2 activity occurred at Mn2+ concentrations of 2–10 mM; also, at these concentrations of Mn2+, Nek2 activity was independent of the presence of Mg2+.

In the absence of divalent cations, Nek2 kinase was inactive. Finally, the presence of the phosphatase inhibitor okadaic acid (1 μM) in extraction and kinase assay buffers stimulated Nek2 activity, suggesting that Nek2 may need to be phosphorylated for maximal activity.

Evidence for Autophosphorylation of Nek2 Protein Kinase—The observed shift in the electrophoretic mobility of Nek2 suggested that human Nek2 was being post-translationally modified in Sf9 cells. In particular, it appeared possible that the retarded electrophoretic mobility of wild-type Nek2 might be the result, at least in part, of autophosphorylation. In support of this view, no molecular weight shift was observed with the catalytically inactive Nek2-K37R mutant (Fig. 2A). To determine whether Nek2 protein was indeed phosphorylated in Sf9 cells in vivo, baculovirus-infected cells were labeled with [32P]orthophosphate and Nek2 as well as Nek2-K37R proteins were immunoprecipitated. As determined by immunoblotting, both proteins were recovered in comparable amounts (Fig. 4A, lower panel); however, only the wild-type Nek2 protein had incorporated detectable amounts of 32P (Fig. 4A, upper panel), indicating that active Nek2 kinase was required for phosphorylation of Nek2 in insect cells. Phosphoamino acid analysis of the in vivo labeled Nek2 protein showed predominantly phosphoserine with a minor contribution of phosphothreonine, but no phosphotyrosine (Fig. 4B). Tryptic phosphopeptide mapping revealed at least eight distinct spots, indicating that the phosphorylation of Nek2 kinase in Sf9 cells is complex (data not shown).

Substrate Specificity of Nek2 Protein Kinase—To probe the substrate specificity of human Nek2 with that of Aspergillus NIMA and murine Nek1, a range of protein and peptide substrates were assayed for their ability to be phosphorylated in vitro by immunoprecipitated Nek2 (for a summary of results, see Table II). In addition to β-casein, Nek2 strongly phosphorylated myelin basic protein and microtubule-associated protein 2 (MAP2); phosphitin, and histone H1 were phosphorylated moderately, whereas enolase, a good in vitro substrate of several tyrosine kinases (24), was not phosphorylated at all. Also, no phosphorylation occurred on bovine serum albumin or immunoglobulin G, and none of the Nek2 substrates was phosphorylated by the Nek2-K37R mutant. Phosphoamino acid analyses were performed on β-casein, myelin basic protein, and MAP2, as well as on in vitro phosphorylated Nek2, and all substrates were found to be phosphorylated exclusively on serine and threonine residues; no phosphotyrosine could be detected even after prolonged exposures (data not shown).

The results of in vitro kinase assays using a series of synthetic peptides are also summarized in Table II. Quantitative analyses of peptide phosphorylation were performed using a phosphocellulose P81 filter binding assay (Fig. 5A), or, where appropriate, TLC followed by autoradiography (Fig. 5B). The peptides chosen for analysis (Fig. 5C) represent preferred substrates for several important families of protein kinases, and most of them have been tested previously for their ability to be phosphorylated by the fungal NIMA protein kinase (21, 25). For control, all peptide phosphorylation experiments were carried out in parallel with both active Nek2 and the inactive Nek2-K37R mutant; none of the peptides studied here was phosphorylated by the catalytically inactive Nek2 kinase (Fig. 5B, and results not shown), ruling out the possibility that contamination of immunoprecipitates by insect kinases might complicate the interpretation of results. Of seven peptides
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TABLE I
Biochemical properties of NIMA-related kinases and casein kinase II

<table>
<thead>
<tr>
<th>Preferred casein subunit substrate</th>
<th>β</th>
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<tr>
<td>pH optima</td>
<td>7.0–9.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Ability to use GTP as phosphate donor</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Inhibited by heparin</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Divalent metal ion requirement</td>
<td>Mn²⁺ &gt;&gt; Mg²⁺</td>
<td>Mg²⁺ &gt;&gt; Mn²⁺</td>
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<tr>
<td>Inhibition by Zn²⁺</td>
<td>Yes</td>
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</tr>
<tr>
<td>Effect of 200 mM NaCl</td>
<td>Inhibitory</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

Fig. 4. In vivo phosphorylation of Nek2 but not Nek2-K37R protein in insect SF9 cells. A, insect SF9 cells were infected with either wild-type Nek2 (lane 2) or Nek2-K37R (lane 1) recombinant baculoviruses and grown in the presence of [³²P]orthophosphate. Nek2 immunoprecipitates were then prepared from cell extracts using the R31 Nek2-specific antiserum and separated by SDS-PAGE before autoradiography (top panel, [³²P]) or immunoblotting with the Nek2-specific monoclonal antibody, NK1 (bottom panel, IB). B, the phosphorylated Nek2 protein was cut out of the dried polyacrylamide gel and subjected to phosphoamino acid analysis. The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards were determined by ninhydrin staining of the TLC plate prior to autoradiography.

TABLE II
Comparison of the substrate specificity of human Nek2 with A. nidulans NIMA and murine Nek1

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Nek2</th>
<th>NIMA</th>
<th>Nek1</th>
</tr>
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<tr>
<td>β-Casein</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>++</td>
<td>+</td>
<td>ND*</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>+++</td>
<td>++</td>
<td>ND</td>
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<tr>
<td>MAP2</td>
<td>+++</td>
<td>++</td>
<td>ND</td>
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<tr>
<td>Histone H1</td>
<td>+</td>
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<tr>
<td>α-Casein</td>
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<td>+</td>
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<tr>
<td>Enolase</td>
<td>–</td>
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<td>Phospholemma (42–72)</td>
<td>+++</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>Phospholemna (65–72)</td>
<td>++</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>S6 kinase peptide</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Kemptide</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Casein kinase peptide</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>cdc2 peptide</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Poly(Glu,Tyr)</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
</tbody>
</table>

* ND, not determined.

tested, three were found to be excellent substrates for human Nek2 kinase (Fig. 5, A and B). Two of these were phospholemna-derived peptides, PLM (42–72) and PLM (65–72); the third was a known preferred substrate of kinases acting on the ribosomal protein S6. Since the short PLM (64–72) peptide contains only one serine and one threonine residue (and no tyrosines), the phosphorylated peptide was recovered from a TLC plate and subjected to phosphoamino acid analysis. Only phosphoserine could be detected (data not shown), demonstrating that the serine in the sequence IRRL-S-TRRR constitutes one phosphoacceptor site for the human Nek2 protein kinase. Indeed, a serine in the context RRL-S-(ST)XR is present in all three Nek2 substrate peptides identified here.

In contrast, three synthetic peptides previously shown to be preferred substrates of cAMP-dependent protein kinase (Leu-Arg-Arg-Ala-Ser-Leu-Gly; Kemptide) (26), casein kinase II (27), and p34cdc2/cyclin B (16), respectively, were not detectably phosphorylated by Nek2 (Figs. 5, A and B). Likewise, as determined by SDS-PAGE on a 15% gel (data not shown), Nek2 did not detectably phosphorylate poly(Glu,Tyr), although this peptide represents a good substrate for many tyrosine kinases and dual-specificity kinases, including murine Nek1 (10). A comparison of the available data on the substrate specificity of the three Nek family members studied so far shows that there is a remarkable similarity in the substrate specificity of fungal NIMA and human Nek2, although the correspondence between the two kinases is not perfect (see Table II and “Discussion”).

Cell Cycle Regulation of Endogenous Nek2 Activity in HeLa Cells—The availability of recombinant Nek2 made it possible to optimize extraction and assay conditions for detecting kinase activity associated with this enzyme. These results set the stage for making another attempt at detecting kinase activity associated with endogenous Nek2 present in cultured human cells. As shown in Fig. 6A, we are now able, for the first time, to detect β-casein kinase activity associated specifically with Nek2 immunoprecipitates (lane 2) but not with control immunoprecipitates (lane 1). Attesting to the specificity of this phosphorylation, it displayed the same characteristics (e.g. insensitivity to heparin; stimulation by Mn²⁺ ions) that had been determined for recombinant Nek2 (data not shown). Phosphorylation of Nek2 itself was also evident in Nek2 immunoprecipitates (Fig. 6A), consistent with the possibility that Nek2 may undergo autophasorylation.

To determine whether Nek2 kinase activity might fluctuate during the cell cycle, HeLa cells were synchronized as described under “Experimental Procedures.” The abundance of Nek2 protein in each sample was determined by immunoblotting (Fig. 6B, upper panel), and Nek2 kinase activity was measured in Nek2 immunoprecipitates, using β-casein as a substrate (Fig. 6B, lower panel). In parallel, the DNA content of each sample was analyzed by flow cytometry (Fig. 6C). In comparison to an exponentially growing, asynchronous population of cells (Fig. 6, B, lane 1, and C, Panel 1), Nek2 activity was low in mitotically arrested (Fig. 6, B, lane 2, and C, Panel 2) and in early G₁ phase cells (Fig. 6, B, lane 3, and C, Panel 3), but high in G₂/M phase cells (Fig. 6B, lane 4, C, Panel 4) and G₂/M phase cells (Fig. 6B, lane 5, and C, Panel 5). In all cases, Nek2 activity levels roughly paralleled the abundance of Nek2 protein (Fig. 6B). These results are consistent with the notion that Nek2 protein kinase may play a role in cell cycle events prior to the onset of mitosis.
DISCUSSION

In this study, we have been able to demonstrate that baculovirus-encoded recombinant Nek2 is active as a serine/threonine-specific protein kinase, and we have carried out an extensive biochemical characterization of this novel enzyme. Nek2 kinase activity can readily be monitored in vitro, using β-casein as an exogenous substrate; alternatively, advantage may be taken of an apparent autophosphorylation activity associated with Nek2. Based on a comparison of various biochemical parameters, as well as a survey of several protein and peptide substrates, we conclude that the human Nek2 protein kinase is remarkably similar to its potential fungal homolog NIMA. In particular, casein was found to be a suitable exogenous substrate for assaying either Nek2 or NIMA. Importantly, however, the casein-kinase activities of Nek2 and NIMA can readily be distinguished from that of the ubiquitously expressed casein kinase II: Nek2 and NIMA specifically target the β- rather than the α-isomer of casein, utilize only ATP but not GTP as a phosphate donor, are unaffected by heparin, and are inhibited rather than stimulated by 200 mM NaCl (see Table 1). One striking difference between Nek2 and NIMA concerns their response to Mn²⁺; whereas Nek2 is stimulated by this divalent cation, NIMA is inhibited (21). Interestingly, in this respect Nek2 resembles Nek1 which was also reported to be stimulated by Mn²⁺ (10).

Both Nek2 and NIMA phosphorylate exclusively serine or threonine residues. Thus, among the NIMA-related kinases studied so far, Nek1 appears to be unique in its ability to phosphorylate tyrosine in addition to serine/threonine, and one may question whether it is appropriate to consider Nek1 and NIMA/Nek2 as members of the same family. We believe, however, that the difference in amino acid specificity between Nek1 and NIMA/Nek2 may be more apparent than real. Although Nek1 clearly displays dual specificity under appropriate experimental conditions (9, 10), it is by no means established that Nek1 will act as a tyrosine kinase under physiological conditions.

So far, we have been unable to purify sufficient quantities of recombinant Nek2 in an active state to warrant an exhaustive analysis of substrate specificity, and the use of immunoprecipitated Nek2 as a source of active kinase has precluded precise determinations of kinetic parameters. Nevertheless, our survey of a number of potential target proteins and peptides provides a first glimpse of the requirements for substrate recognition by Nek2. Whereas the precise sites phosphorylated by Nek2 have not been mapped for most of the proteins studied here, we found that the peptide IRRRLSTRRR was phosphorylated exclusively on serine, suggesting that basic residues may contribute to substrate recognition by Nek2. In support of this view, the Nek2 protein contains acidic residues within the kinase domain which, based on homology to protein kinase A (22, 28, 29), would suggest a requirement for arginine residues at positions −2 and −3 in the target sequence. However, since Kemptide also contains N-terminal arginines, yet is not a substrate of Nek2, it is likely that basic residues C-terminal to the phosphorylation site may also be important for substrate recognition by Nek2. More systematic studies on synthetic peptides with permutations at various positions would be necessary to more definitively assess the relevance of individual residues within the above sequence.

Interestingly, recent studies on NIMA have suggested a requirement for a phenylalanine N-terminal to the phosphorylated serine or threonine; hence, the motif FXST was proposed to constitute a consensus for a NIMA phosphorylation site (25). The results reported here indicate that this purported phenylalanine requirement cannot be extended to Nek2 (see Fig. 5 and Table II). However, we note that the proposed NIMA consensus sequence is clearly not absolute, since several good substrates of NIMA, including β-casein and NIMA itself, do not contain this sequence.

Finally, and perhaps most importantly, the present study has allowed us to identify conditions under which the kinase activity of endogenous Nek2 can now be assayed in immunoprecipitates prepared from human cells. This in turn made it possible to carry out a first analysis of the regulation of Nek2
Nek2 Kinase Activity

may play a role in cell cycle events leading to the onset of mitosis. A similar role has also been proposed for fungal NIMA (2), but in contrast to Nek2 (this study), NIMA has been reported to be maximally active in mitotically arrested cells (13). This difference does not exclude that NIMA and Nek2 might carry out related biological functions, since the temporal organization of certain cell cycle events (e.g. spindle formation) is known to differ between fungi and mammals, but it raises the possibility that vertebrate Nek2 may be required already at an earlier cell cycle stage than fungal NIMA. Physiological substrates remain to be identified for both NIMA and Nek2, and definitive judgment on the potential functional homology between these kinases must await discovery of such substrates.

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REFERENCES

Fig. 6. Nek2 activity fluctuates during the cell cycle in HeLa cells. A, immunoprecipitates were prepared from asynchronous HeLa cells, using either preimmune (lane 1, P) or anti-Nek2-specific immune serum (lane 2, I). Kinase assays were then carried out in the presence of casein, as indicated under "Experimental Procedures" (optimized conditions). Proteins were subjected to SDS-PAGE and autoradiography. Molecular masses are indicated on the left, and the positions of Nek2 and β-casein are marked on the right. B, HeLa cell extracts were prepared from an exponentially growing population (lane 1), from cells blocked with nocodazole (lane 2) or aphidicolin (lane 4), and from cells that had been released for 6 h from the nocodazole block (lane 3) or the aphidicolin block (lane 5). Samples were equilibrated for protein content and subjected to SDS-PAGE and immunoblotting (IB) (upper panel) or immunoprecipitation, using the anti-Nek2 antibody. Immunoprecipitates were then used for kinase assays carried out in the presence of [γ-32P]ATP and exogenous β-casein as a substrate. Reaction products were analyzed by SDS-PAGE and autoradiography (lower panel; [32P]). The positions of Nek2 and β-casein are indicated. C, a portion of each cell population used for obtaining the data in Panel B was processed for the determination of DNA content by FACS analysis, as described under "Experimental Procedures." For each sample, the positions of the G1 phase peak and the G2/M phase peak are marked by arrowheads.