Mitotic Reorganization of the Intermediate Filament Protein Nestin Involves Phosphorylation by cdc2 Kinase*

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The intermediate filament protein nestin is expressed during early stages of development in the central nervous system and in muscle tissues. Nestin expression is associated with morphologically dynamic cells, such as dividing and migrating cells. However, little is known about regulation of nestin during these cellular processes. We have characterized the phosphorylation-based regulation of nestin during different stages of the cell cycle in a neuronal progenitor cell line, ST15A. Confocal microscopy of nestin organization and 32P in vivo labeling studies show that the mitotic reorganization of nestin is accompanied by elevated phosphorylation of nestin. The phosphorylation-induced alterations in nestin organization during mitosis in ST15A cells are associated with partial disassembly of nestin filaments. Comparative in vitro and in vivo phosphorylation studies identified cdc2 as the primary mitotic kinase and Thr316 as a cdc2-specific phosphorylation site on nestin. We generated a phosphospecific nestin antibody recognizing the phosphorylated form of this site. By using this antibody we observed that nestin shows constitutive phosphorylation at Thr316, which is increased during mitosis. This study shows that nestin is reorganized during mitosis and that cdc2-mediated phosphorylation is an important regulator of nestin organization and dynamics during mitosis.

The intermediate filament (IF) protein nestin is expressed during early stages of development in progenitor cells of the central nervous system. Nestin is also found in developing muscle and myocardial cells (1–4) as well as in developing sertoli cells (5). Upon differentiation, nestin expression is down-regulated and replaced by other tissue-specific IF proteins, such as glial fibrillary acidic protein (GFAP) in astrocytes, neurofilaments in neurons, and desmin in muscle tissue, respectively. Interestingly, nestin expression is reinduced during various regenerative and degenerative conditions in the fully differentiated organism (6, 7).

Coexpression of nestin and the class III IF proteins, vimentin, GFAP, and desmin, has been documented in developing neuronal and embryonic cells. In these cells, nestin shows a similar intracellular organization as the class III IF proteins (1, 8). Based on these observations, a possible copolymerization of nestin and the class III IF proteins, has been postulated. Recent data showed that nestin fails to assemble into filaments in the absence of a coexpressed IF network in astrocytes (9). In addition, it was recently shown that nestin cannot polymerize on its own but is integrated into type III IFs, most likely as a heterodimer (10).

Nestin has been shown to be associated with dividing and migrating cells and with cells rapidly changing their morphology. In light of the fact that nestin is expressed particularly in dynamic cells, it is of interest to study posttranslational modifications of nestin and the involvement of such modifications in regulating nestin organization. Intermediate filament proteins form dynamic structures that change their intracellular organization during various conditions, such as mitosis, differentiation, and different pathological situations (reviewed in Refs. 11–15). The regulatory mechanisms behind these changes are still not fully understood, but phosphorylation has been implicated as an important regulator of IFs (reviewed in Refs. 13 and 16–18). The role of phosphorylation in regulating IF organization is supported by studies showing that several kinases, including protein kinase C, eAMP-dependent kinase, Ca2+/calmodulin-dependent protein kinase, and cdc2 kinase, induce disassembly of IFs in vitro (18–21). Changes in IF morphology can also be induced by elevating kinase activities in vivo, for example by expression of constitutively active forms of protein kinase C or Ca2+/calmodulin-dependent protein kinase II in astrocytes (22) or by microinjection of the catalytic subunit of protein kinase A into fibroblasts (23). Several IF proteins have been shown to undergo increased phosphorylation with subsequent reorganization of IF structure during mitosis. These proteins include vimentin (24, 25), GFAP (26, 27), keratins 8 and 18 (28, 29), and the nuclear lamins (30, 31). cdc2 kinase has been identified as a key regulator of IF organization during mitosis (reviewed in Refs. 13 and 18). Phosphorylation may also be involved in regulating the specific cellular distribution of IFs, as demonstrated by the preferential localization of phosphorylated neurofilaments in axons (32, 33), and...
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The localization of K18, phosphorylated on Ser252, to different cellular domains depending on the tissue (34). In addition to changes in IF protein phosphorylation during mitosis, there appears to be a continuous phosphate turnover on IFs in interphase, which could be involved in regulating the dynamic properties of IFs in interphase cells (13, 16, 35, 36). Phosphorylation has also been implicated in the regulation of the interactions of IFs with both cytoskeleton-associated proteins, e.g. plectin, desmoplakin (37, 38), or signaling proteins, e.g. 14-3-3 (39–41).

The intracellular organization of nestin has not been characterized in detail, and little is known about the structural regulation of nestin during different cellular processes. In view of the previous observations on mitotic vimentin phosphorylation by cdc2, we have characterized the role of cdc2 kinase in regulating nestin organization during mitosis in an immortalized central nervous system precursor rat cell line, ST15A. In addition, we have identified Thr316 as an in vitro cdc2-specific nestin phosphorylation site. We have generated a phospho-specific nestin antibody against the phosphorylated Thr316 peptide to characterize phosphorylation of nestin at this site in vivo. The phosphorylation of this site is elevated during mitosis but also shows a certain degree of constitutive phosphorylation in interphase cells.

MATERIALS AND METHODS

Cell Culture and Synchronization—Immortalized rat central nervous system precursor cells, ST15A (42), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator in a 5% CO2 atmosphere at 37 °C. For synchronization, cells were arrested in mitosis with 0.3 μg/ml nocodazole (Sigma) for 12 h. The mitotic cells were harvested by mechanical shake off, washed three times in PBS, before centrifugation and harvesting. The protein concentrations were determined by Bradford assay or by densitometric scanning of SDS-PAGE-separated protein bands, and the samples were normalized before immunoprecipitation or immunoblotting.

Antibodies, Double Immunofluorescence, and Confocal Microscopy—For double label immunofluorescence experiments, cells were grown on coverslips coated with 0.02 mg/ml poly-L-lysine (Sigma). Cells were washed in phosphate-buffered saline (PBS) and fixed in 100% methanol for 10 min at −20 °C. Cells were rinsed in PBS, blocked with 1% bovine serum albumin, and incubated with nestin antiserum 130 diluted 1:500 or nestin antiserum 6 diluted 1:200 for 30 min at room temperature. The cells were then rinsed three times in PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (Molecular Probes). Cells were washed with PBS and further incubated with a mouse monoclonal anti-vimentin antibody (RPN 1102, Amersham) diluted 1:50, and washed three times in PBS before mounting in Mowiol (Amerham Pharmacia Biotech), to which the synthesized phosphopeptide and unphosphorylated peptide, respectively, were covalently coupled. For immunoblotting the double affinity purified anti-phospho-Thr316 nestin serum was diluted 1:2000 in 5% bovine serum albumin/MOPS buffer (50 mM MOPS, 125 mM NaCl, and 1.2 mM NaOH, pH 7.4) and incubated overnight at 4 °C. The blots were washed four times in MOPS buffer supplemented with 0.3% Tween, before incubation with an horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Blots were developed using the ECL Western blotting detection system (Amersham Pharmacia Biotech). To test the specificity of the antiserum, it was preincubated with excess phosphorylated peptide before incubating the blots. For immunofluorescence, the anti-Thr316 nestin serum was diluted 1:50 in 1% bovine serum albumin/PBS and incubated overnight at 4 °C, then washed three times with PBS, and further incubated with secondary antibody. Phosphorylation of vimentin on its cdc2-specific site, Ser55, was detected as previously described by using a phosphopeptide-specific antibody against vimentin phosphorylated on Ser55 (44).

Determination of Nestin Phosphorylation Levels and Identification of Nestin Phosphopeptides—ST15A cells were metabolically labeled with 300 μCi/ml [32P]orthophosphate for 4 h in phosphate-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum in the presence of 0.3 μg/ml nocodazole. This procedure blocked the cells entering mitosis. Mitotic cells were harvested by mechanical shake-off, centrifuged down at 1500 × g 5 min at +4 °C, and washed twice by centrifugation in ice-cold PBS. Adherent cells were washed once with ice-cold PBS and harvested by scraping. Whole cell extracts were obtained by lysing the cells in SDS lysis buffer (20 mM Tris-HCl, pH 7.2, 5 mM EGTA, 5 mM EDTA, 0.4% SDS, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml antipain, and 10 μg/ml pepstatin, respectively, and 1 mM PMFS). The cell extracts were boiled for 5 min and further sonicated for 20 s with a probe sonicator. Nestin and vimentin were immunoprecipitated by dilution of whole cell extracts with RIPA buffer (20 mM Hepes, pH 7.4, 140 mM NaCl, 10 mM pyrophosphate, 5 mM EDTA, 0.4% Nonidet P-40, 100 mM PMFS, 10 μg/ml leupeptin, and 10 μg/ml antipain) added to either anti-nestin antibody 130 or rabbit vimentin antibody 264, respectively, and finally recovered with protein A-Sepharose (Sigma).

The immunoprecipitated proteins were separated on SDS-polyacrylamide gels (45) and stained with either Coomassie Brilliant Blue or silver to control for equal loading of proteins. The gels were dried and autoradiographed at −70 °C using Kodak X-Omat AR or Kodak Biomax MS films. Specific [32P] labeling of proteins was quantified using a phosphomager (Bio-Rad).

For phosphopeptide or phosphoamino acid analysis, vimentin and nestin were separated on 7.5% SDS-PAGE, fixed in 50% methanol, dried, and autoradiographed. The corresponding nestin and vimentin bands were cut out from the gels digested twice (9 + 3 h) with trypsin (T-8642, Sigma, 10 μg/ml in 50 mM ammonium bicarbonate) at 37 °C. The digested peptides were washed with double-distilled water and dried using a speed vac. Peptide maps of trypsin-digested samples (46) were obtained by two-dimensional separation on microcrystalline cellulose TLC plates (Merek). Trypsinized peptides were also subjected to...
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phosphoamino acid analysis by acid hydrolysis and further two-dimensional TLC electrophoresis together with Ser(P), Tyr(P), and Thr(P) standards (46). Phosphoamino acids were visualized with ninhydrin staining and autoradiography.

Separation of IF Fractions Following Extraction with Triton X-100— Triton X-100 buffer (20 mM Hepes, pH 7.6, 100 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml antipain) was added to culture dishes, cells were detached and collected, homogenized on ice, and centrifuged for 15 min at 10,000 × g (4°C) to pellet IF. The supernatant was further centrifuged at 200,000 × g for 30 min at 4°C to obtain a soluble fraction (supernatant) and a pellet containing additional, most likely fragmented IF. All fractions were dissolved in 3× Laemmli sample buffer (45) for further gel electrophoresis and Western blotting.

In Vitro Phosphorylation of Nestin and Vimentin and Identification of cdc2-specific Phosphorylation Sites on Nestin—For isolation of cellular IF, ST15A cells were harvested in lysis buffer (0.6 M KCl, 2 mM for isolation of cellu-


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soluble fraction after 10,000 g centrifugation. The figure shows immunoblotting of Triton-X-insoluble fraction after 10,000 g, insoluble and soluble fractions after 200,000 g of control, and nocodazole-treated ST15A cells. Blocking ST15A cells in mitosis with nocodazole increases the 200,000 g insoluble fraction after 10,000 g in nocodazole-treated cells. However, there was an increase in the soluble fraction after centrifugation at 200,000 g (Fig. 3), indicating the presence of disassembled soluble subunits. Note the presence of the lower molecular mass protein bands, representing cleaved forms of nestin. The intensity of these bands increases in the soluble fractions of nocodazole-treated cells, indicating a possible processing of nestin during mitosis.

Mitotic Phosphorylation of Nestin Is Mediated by cdc2 Kinase—The mitotic reorganization of the cytoplasmic IF networks in many cell types is known to be regulated by phosphorylation of the IF proteins. Therefore, the phosphorylation state of nestin in ST15A cells during interphase-mitosis transition was examined. The mitotic reorganization of the cytoplasmic IF networks in ST15A cells was accompanied by an increase in the phosphorylation levels of the IF proteins, nestin and vimentin (Fig. 4A). In accordance with previous studies (24, 25), vimentin showed a low level of constitutive phosphorylation, which was elevated 6-fold in mitotic cells (Fig. 4A, left panel). In comparison with vimentin, nestin had a higher level of constitutive phosphorylation in interphase cells and showed a 3-fold increase in the phosphorylation level during mitosis (Fig. 4A, right panel). In addition to the ~220-kDa nestin band, immunoprecipitation with the nestin antibody also yielded lower molecular mass bands. These bands were weakly phosphorylated in control cells and showed increased phosphorylation in nocodazole-treated cells. They most likely represent cleaved forms of nestin, because immunoblotting of IF preparations from ST15A cells (Fig. 1) and whole cell extracts of ST15A cells (data not shown) using antibodies against nestin shows the presence of corresponding bands. Phosphoamino acid analysis of nestin immunoprecipitated from in vivo 32P-labeled interphase and mitotic preparations as compared with interphase samples (5). Although not obvious from Fig. 5, which does not give a quantitative presentation of the relative labeling intensity on interphase versus mitotic peptides, peptide 5 showed consistently elevated phosphorylation in mitotic preparations as compared with interphase samples. According to quantification by phosphoimager image analysis, the relative increase (mitosis/interphase) was 1.9-fold ± 0.3 (mean ± range of values; measurements made on peptide maps obtained from five different experiments). The mitosis-specific phosphopeptides 1, 2, 3, 4, and 6 corresponded
Using a polyclonal antibody raised against the carboxyl terminus of nestin (rabbit anti-nestin 6) showed a similar pattern (Fig. 7B, third panel). The polyclonal antibody recognized the ~175-kDa band in addition to prominent immunoreactivity against the ~220-kDa band (Fig. 7B, third panel). Immunoblotting of whole cell extract of ST15A cells with a monoclonal nestin antibody also gave immunoreactivity at ~175 kDa (Fig. 7B, fourth panel).

The results by using the phosphopeptide antibody indicated that nestin was constitutively phosphorylated at Thr\textsuperscript{316} and that phosphorylation at this site increased during mitosis, as shown by the increased intensity of the ~220- and the ~175-kDa bands in nocodazole-treated cell samples (Fig. 7B, first panel). The phosphorylation of the ~175-kDa band was especially pronounced. Experiments on competitive binding inhibition, where the antibody was preincubated with phosphopeptide in excess, almost completely abolished the antibody reactivity of the ~220-kDa band and the band at ~175-kDa (Fig. 7B, second panel from the left). Immunoblotting of \textit{in vitro} cdc2-phosphorylated and unphosphorylated IF preparations showed that cdc2 kinase increases the phosphorylation of nestin (Fig. 7C).

Immunoblotting with a monoclonal nestin antibody of insoluble and soluble fractions of control and nocodazole-treated cells after centrifugation at 10,000 \( \times g \) and 200,000 \( \times g \) also revealed the presence of the ~175-kDa band (Fig. 3). This band seems to transfer to the soluble pool after centrifugation at 200,000 \( \times g \) during mitosis, because the intensity of the band increases in the soluble fraction of nocodazole-treated cells. Another possible explanation for the increased intensity is that nestin is cleaved during mitosis to yield an increased amount of a soluble ~175-kDa peptide.

The constitutive phosphorylation of nestin at Thr\textsuperscript{316} in interphase cells and the increase in phosphorylation at this site during mitosis was further supported by immunolabeling of interphase and mitotic cells using the phosphospecific antibody (Fig. 7D). Confocal images showed weak immunoreactivity on nestin IFs in interphase cells with an elevated reactivity in mitotic cells. In interphase cells there is a punctate staining along the filaments. Toward the cell periphery the staining becomes more diffuse. The immunoreactivity is strongest around the nucleus. In mitotic cells the staining is more intense, but no clear filaments are seen, compared with the monoclonal nestin staining, where filaments can be visualized. In comparison, the one identified cdc2-target on vimentin, i.e. Ser\textsuperscript{52}, showed complete mitotic specificity with no phosphorylation in interphase cells, as revealed with a Ser(P)\textsuperscript{52}-specific antibody (Fig. 7D).

DISCUSSION

During interphase, nestin and vimentin form indistinguishable cytoplasmic networks in ST15A cells. This indicates that these two IF proteins form copolymers. Copolymerization between nestin and class III IF proteins has been discussed in earlier studies (1, 8) and is supported by the findings that neither nestin nor vimentin can form filaments in GFAP-deficient astrocytes without the presence of a coexpressed IF network (9). The ability of nestin to coassemble with vimentin in \textit{vitro} has recently been shown (10). A model for coassembly of nestin and vimentin was proposed, where nestin-vimentin heteropolymers are added to a core of vimentin homopolymers, allowing the long carboxyl-terminal of nestin to stick out of the filament body. This idea was further developed in a recent review where it was proposed that nestin could function as a linker protein, where the long tail of nestin would interact with the microfilaments and the microtubules, interconnecting the three distinct components of the cytoskeleton (49). According to

\[ \text{\textsuperscript{2}C. M. Sahlgren and J. E. Eriksson, unpublished observations.} \]
our results, there is a dramatic reorganization of the IF networks into cage-like structures in normal and nocodazole-arrested mitotic ST15A cells. Coincident with the morphological changes seen in the IF networks during mitosis in ST15A cells, there is an increase in the phosphorylation levels of nestin and vimentin. Nestin shows constitutive phosphorylation in interphase cells with a 3-fold increase in mitotic cells. In comparison, the elevated phosphorylation of mitotic vimentin is more dramatic, about 6-fold. The constitutive phosphorylation of nestin may correspond to specific phosphorylation sites involved in the tissue-specific functions of nestin. Other possible functions for phosphorylation of nestin are regulation of the spatial organization of IF proteins, as has been shown for neurofilaments, or regulation of protein-protein interactions with other IF proteins, such as vimentin. Assuming that nestin coassembles with type III IF proteins in vivo, it is possible that the long carboxyl-terminal domain of nestin functions as a spacer or cross-linking element between IFs and other cytoskeletal components, thereby regulating their supramolecular organization as proposed in Refs. 10 and 49. Phosphorylation could modulate the configuration of the tail domain, affecting the formation of protein-protein interactions and thereby the organization of the cytoskeletal network. In this study, the data suggest a role for cdc2 kinase in the phosphorylation of nestin and its reorganization during mitosis. Furthermore, we identified Thr316 as a cdc2-specific in vitro phosphorylation site of nestin. Interestingly, a corresponding ~175-kDa band could be seen in autoradiographs of nestin immunoprecipitated from in vivo labeled mitotic ST15A cells. Phosphorylation of this band was increased in nocodazole-treated cells (Fig. 2). There is a difference between nestin and vimentin when it comes to proline-directed phosphorylation. The only SP site on vimentin (Ser55) shows no constitutive phosphorylation, whereas the TP site on nestin (Thr316) shows significant phosphorylation in interphase cells. The kinase responsible for the constitutive phosphorylation at Thr316 is unknown, but it could be another cyclin-dependent kinase. For example, cdk5, which is required for normal neuronal differentiation and muscular development (50–52), might be a candidate for interphase-specific phosphorylation of nestin at Thr316. cdk5 together with p35, the protein activator of cdk5, plays a role in the regulation of the structure of the neuronal cytoskeleton structure because it has been shown to phosphorylate cytoskeletal components such as the microtubule-associated protein tau and the neuronal IF protein, neurofilament H (53, 54). Considering the requirement for cdk5 activity for proper neuronal and muscular development and the effect of cdk5 phosphorylation on cytoskeletal organization, we tested whether this kinase can phosphorylate nestin in vitro, and our preliminary data shows that nestin is a bona fide

![Graph](image-url)
substrate for cdk5. cdk5-mediated phosphorylation could explain the phosphorylation observed in interphase cells, but further studies are required to elucidate this.

Our data indicate that cdc2 kinase is involved in the regulation of nestin organization during mitosis. It remains to be determined whether phosphorylation by cdc2 is sufficient to account for the reorganization of nestin during mitosis or whether some other kinase is required. There are other kinases in addition to cdc2 kinase that have been shown to phospho-
ylate IF in vivo during mitosis, including p37 kinase, which phosphorylates vimentin at Ser558 and Thr147 in mitotic BHK-21 cells (55); protein kinase C, which shows mitosis-specific phosphorylation of lamin B and vimentin (56, 57); and Rho kinase, which phosphorylates GFAP during late mitosis (57, 58). Recently, Rho kinase was shown to phosphorylate vimentin at Ser73 at the cleavage furrow in late mitotic cells (60).

In summary, our interest in IF dynamics has led us to study

FIG. 7. Characterization of nestin phosphorylation at Thr316 in vivo. A, phosphopeptide-specific antibody against phosphorylated Thr316 was generated. A, immunoblotting of 0.5 μg of the Thr316 phosphorylated and unphosphorylated peptide spotted on nitrocellulose membrane revealed specificity of the generated antibody. The antibody recognizes the phosphorylated peptide but shows no immunoreactivity against the unphosphorylated peptide. B, based on immunoblotting of control and nocodazole-treated cells, nestin is constitutively phosphorylated at this site during interphase, but the phosphorylation level is increased during mitosis. The phospho-specific peptide antibody shows strong immunoreactivity against a ~175-kDa nestin peptide, in addition to reactivity against the full-length ~220-kDa nestin (P-thr-316 nestin). The same samples were immunoblotted with a polyclonal nestin antibody (anti-nestin 6) to confirm the identity of the ~175-kDa band (polyclonal anti-nestin). Immunoblotting of whole cell extracts of ST15A cells with a monoclonal nestin antibody also showed the presence of the ~175-kDa peptide (monoclonal anti-nestin). Preincubating the antibody with the phosphorylated peptide abolished immunoreactivity against the ~220-kDa band and the lower molecular mass band of ~175 kDa (P-thr-316 nestin + p-peptide). The additional low molecular bands seen on the blot were still visible when the antibody was preincubated with excess of the phosphorylated peptide. Equal numbers of control and nocodazole-treated cells were harvested, and equal loading of proteins was further controlled by Coomassie Brilliant Blue staining (data not shown). C, immunoblotting of in vitro cdc2-phosphorylated and unphosphorylated IF preparations shows that cdc2 kinase increases phosphorylation at Thr316. D, double immunofluorescence labeling of ST15A cells with the Thr316-nestin phosphopeptide-specific antibody and monoclonal nestin antibody. Immunofluorescence labeling of interphase ST15A cells with the nestin phosphopeptide specific antibody (panel a) shows a fragmented pattern along the filaments. The intensity of the staining is increased in mitotic cells. As comparison, panel b shows labeling with the monoclonal nestin antibody, where the labeling in interphase versus mitotic cells is of similar intensity. Panel c shows labeling of ST15A cells with a phosphopeptide antibody which recognizes vimentin when phosphorylated on the major cdc2-specific site, Ser57. Panel d shows the whole vimentin IF network as revealed by a monoclonal vimentin antibody. Immunoreactivity of the vimentin phosphopeptide-specific antibody is absent in interphase cells.

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the previously uncharacterized regulation of the IF protein nestin during the cell cycle. We have shown that reorganization of nestin during mitosis is coupled to increased phosphorylation of nestin and that cdc2 kinase seems to play a major role in this phosphorylation-mediated structural modification of nestin.

Acknowledgments—We want to thank Igor Bryzgalov for assistance in generating the antibodies and Minna Poukkula for critical comments on the manuscript. We are also grateful to and Kaija-Liisa Laine and Helena Saarento for technical assistance.

REFERENCES

1. Lendahl, U., Zimmerman, L., and McKay, R. D. G. (1990) J. Histochem. Cytochem. 38, 833–847
2. Zimmerman, L. B., Lendahl, U., Cunningham, M., McKay, R. D. G., Pau, B., Gavrin, B., Mann, J., Vassileva, G., and McMahon, A. (1994) Neuron 12, 11–24
3. Sejersen, T., and Lendahl, U. (1993) J. Cell Sci. 106, 1291–1300
4. Fitchett, E., and Cleveland, D. W. (1998) J. Biol. Chem. 273, 514–519
5. Ku, N.-O., Liao, J., and Omary, B. M. (1994) Adv. Prot. Phosphatases 7, 1892–1906
6. Eriksson, J. E., Opal, P., and Goldman, R. D. (1992) Curr. Opin. Cell Biol. 4, 99–104
7. Tzivion, G., Luo, Z. J., and Avruch, J. (2000) J. Biol. Chem. 275, 29772–29778
8. Ku, N.-O., Liao, J., and Omary, B. M. (1998) EMBO J. 17, 1892–1896
9. Geisler, N., Hatzfeld, M., and Weber, K. (1989) J. Cell Biol. 109, 1063–1071
10. Chae, T., Kwon, Y. T., Bronson, R., Dikkes, P., Li, E., and Tsai, L. H. (1997) EMBO J. 16, 3474–3488
11. Ku, N.-O., Liao, J., and Omary, B. M. (1998) EMBO J. 17, 1892–1896
12. Klymkowsky, M. W. (1996) J. Cell Biol. 131, 1291–1301
13. Ku, N.-O., Liao, J., and Omary, B. M. (1998) EMBO J. 17, 892–896
14. Geisler, N., and Cleveland, D. W. (1998) Science 279, 514–519
15. Ku, N.-O., Liao, J., and Omary, B. M. (1998) EMBO J. 17, 2409–2422
16. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 2409–2422
17. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
18. Ku, N.-O., Liao, J., and Omary, B. M. (1998) EMBO J. 17, 892–896
19. Ku, N.-O., Liao, J., and Omary, B. M. (1998) EMBO J. 17, 892–896
20. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
21. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
22. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
23. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
24. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
25. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
26. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
27. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
28. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
29. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
30. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
31. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
32. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
33. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
34. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
35. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
36. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
37. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
38. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
39. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
40. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
41. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
42. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
43. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
44. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
45. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
46. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
47. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
48. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
49. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
50. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
51. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
52. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
53. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
54. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
55. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
56. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
57. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
58. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
59. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
60. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
61. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
62. Ku, N.-O., and Omary, B. M. (1994) J. Biol. Chem. 269, 514–519
Mitotic Reorganization of the Intermediate Filament Protein Nestin Involves Phosphorylation by cdc2 Kinase
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J. Biol. Chem. 2001, 276:16456-16463.
doi: 10.1074/jbc.M009669200 originally published online February 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M009669200

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