Multisite Phosphorylation of Nuclear Interaction Partner of ALK (NIPA) at G₂/M Involves Cyclin B1/Cdk1*‡§¶

Florian Bassermann†1, Christine von Klitzing‡, Anna Lena Illert‡, Silvia Münch‡, Stephan W. Morris§, Michele Pagano*, Christian Peschel† and Justus Duyster‡12

From the †Department of Internal Medicine III, Technical University of Munich, 81675 Munich, Germany, the ‡Departments of Pathology and Hematology-Oncology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105, and the §Department of Pathology and New York University (NYU) Cancer Institute, NYU School of Medicine, New York, New York 10016

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Nuclear interaction partner of ALK (NIPA) is an F-box-containing protein that defines a nuclear skp1 cullin F-box (SCF)-type ubiquitin E3 ligase (SCF(NIPA)) implicated in the regulation of mitotic entry. The SCF(NIPA) complex targets nuclear cyclin B1 for ubiquitination in interphase, whereas phosphorylation of NIPA in late G₂ phase and mitosis inactivates the complex to allow for accumulation of cyclin B1. Here, we identify the region of NIPA that mediates binding to its substrate cyclin B1. In addition to the recently described serine residue 354, we specify 2 new residues, Ser-359 and Ser-395, implicated in the phosphorylation process at G₂/M within this region. Moreover, we found cyclin B1/Cdk1 to phosphorylate NIPA at Ser-395 in mitosis. Mutation of both Ser-359 and Ser-395 impaired effective inactivation of the SCF(NIPA) complex, resulting in reduced levels of mitotic cyclin B1. These data are compatible with a process of sequential NIPA phosphorylation where cyclin B1/Cdk1 amplifies phosphorylation of NIPA once an initial phosphorylation event has dissociated the SCF(NIPA) complex. Thus, cyclin B1/Cdk1 may contribute to the regulation of its own abundance in early mitosis.

The SCF family of E3 ubiquitin ligases essentially regulates the abundance of key cell cycle regulatory proteins. The F-box protein is the only variable component of the SCF complex that mediates substrate binding and thus determines specificity of the respective SCF complex (1–4). To bind the respective ubiquitination target, each F-box protein contains a protein interaction domain. Depending on the type of the interaction domain, F-box proteins have been classified into three major classes; two classes of F-box proteins contain WD40 repeats and leucine-rich repeats (5, 6), whereas a third class contains other types of protein interaction domains or no canonical leucine-rich repeats (5, 6).

We previously reported the cloning of NIPA (nuclear interaction partner of ALK) and subsequently characterized NIPA as an F-box-containing protein that defines an ubiquitin E3 ligase (SCF(NIPA)) that targets nuclear cyclin B1 in interphase, thus contributing to the timing of mitotic entry (8–10). The oscillating activity of the SCF(NIPA) complex is governed by cell cycle-dependent inhibitory phosphorylation of NIPA in late G₂ phase that dissociates NIPA from the SCF core. This phosphorylation event was found to occur, at least in part, on Ser-354 (9). The identity of the kinase(s) responsible for NIPA phosphorylation at G₂/M and during mitosis has so far remained elusive.

The substrate of NIPA, cyclin B1, together with its associated kinase Cdk1, form the maturation-promoting factor that essentially regulates the transition from G₂ phase into mitosis. Although mitotic entry requires activity of cyclin B1/Cdk1, mitotic exit requires its destruction. At mitotic entry, regulation of the maturation-promoting factor occurs at two distinct levels: the regulated phosphorylation of the Cdk1 subunit and the nuclear abundance of cyclin B1. In the case of Cdk1, inhibitory phosphates are regulated by the kinase Wee1 and the phosphatase Cdc25 (11–13). The timely abundance of cyclin B1 in the nucleus is procured by G₂ phase-specific phosphorylation of cyclin B1 that mediates nuclear translocation and the timely termination of nuclear cyclin B1 destruction mediated by the SCF(NIPA) complex (9, 14–16). In late mitosis, control of cyclin B1 is governed by the anaphase-promoting complex/cyclosome (APC/C) that targets cyclin B1 for proteasomal destruction to allow for subsequent mitotic exit (17–19). A feature of cyclin B1/Cdk1 marks its ability to control its own regulators. At the G₂/M transition and early mitosis, cyclin B1/Cdk1 has been shown to further activate Cdc25 via phosphorylation and to induce Wee1 degradation (20–22). Moreover, cyclin B1/Cdk1 regulates mitotic APC/C activity.

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† To whom correspondence may be addressed: Dept. of Pathology and NYU Cancer Institute, NYU School of Medicine, 550 First Ave., NY, NY 10016. Tel.: 212-263-5129; Fax: 212-263-5107; E-mail: florian.bassermann@med.nyu.edu.

‡ To whom correspondence may be addressed: Dept. of Internal Medicine III, Technical University of Munich, Ismaningerstrasse 22, D-81675 Munich, Germany. Tel.: 49-89-4140-4104; Fax: 49-89-4140-4879; E-mail: justus.duyster@lrz.tum.de.

§ The abbreviations used are: SCF, skp1 cullin F-box; NIPA, nuclear interaction partner of ALK; NPM-ALK, nucleophosmin-anaplastic lymphoma kinase; Cdk, cyclin-dependent kinase; CSF, cytostatic factor; APC/C, anaphase-promoting complex/cyclosome; FBX, F-box; GST, glutathione S-transferase; WT, wild type; GSK3β, glycogen synthase kinase-3.
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via phosphorylation of the APC and its subunits Cdc20 and Cdh1 to control the sequential activation of APC\textsuperscript{Cdc20} and APC\textsuperscript{Cdh1} (19, 23–26).

To further classify the F-box protein NIPA, we have attempted to identify the protein interaction region of this molecule that mediates binding to its substrate cyclin B1. We identify a 50-amino-acid region spanning from amino acids 352–402 in the C terminus of NIPA as the relevant binding determinant. Next to Ser-354, we found 2 new residues within 402 in the C terminus of NIPA as the relevant binding determinant. Since phosphorylation is necessary for efficient dissociation of the SCF\textsuperscript{NIPA} in late G\textsubscript{2} and mitosis. Moreover, we show that cyclin B1/Cdk1 phosphorylates NIPA at Ser-395 in mitosis. These findings suggest that cyclin B1/Cdk1 contributes to the efficient mitotic inactivation of the SCF\textsuperscript{NIPA} complex in terms of a negative feedback loop and may thus contribute to the regulation of its own nuclear abundance in early mitosis.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Immunological Procedures—Details of the construction of the various NIPA plasmids are available from the authors upon request. Point mutations of the NIPA cDNA were engineered using the QuickChange mutagenesis kit (Stratagene), whereas deletion mutants were prepared by PCR using standard cloning procedures. Mouse monoclonal antibodies were purchased from Sigma (anti-FLAG (M2), anti-Myc (clone 9E10), anti-β-actin). Polyclonal rabbit antibodies were from Santa Cruz Biotechnology (anti-cyclin A, anti-cyclin B1, anti-Skp1) and Zymed Laboratories Inc. (anti-cull1). Immunoblot analysis and immunoprecipitations were performed as described (27).

Cell Culture and Cell Cycle Analysis—HeLa, Cos1, and 293T cells were cultivated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transient transfections of HeLa, 293T, and Cos1 cells were performed using FuGENE 6 (Roche Applied Science), Lipofectamine 2000 (Invitrogen), or Dotap (Roche Applied Science) transfection reagents, respectively. Stable transfections of HeLa cells were performed using the FuGENE 6 (Roche Applied Science), Lipofectamine 2000 (Invitrogen), or Dotap (Roche Applied Science) transfection reagents, respectively. Stable transfections of HeLa cells were performed using the FuGENE 6 (Roche Applied Science) reagent and subsequent selection with Zeocin\textsuperscript{®} at 100 µg/ml. Cell synchronization at G\textsubscript{2}/M was performed by sequential culture with 2 mM thymidine and 40 ng/ml nocodazole and subsequent collection of rounded cells. Cell cycle distribution was determined by flow cytometry and subsequent analysis using the FlowJo software (Tree Star Inc., Ashland, OR).

GST Fusion Proteins and Pull-down Assays—NIPA WT and all NIPA point and deletion mutants were expressed in Escherichia coli using pGEX vectors (Amersham Biosciences). For protein purification, bacteria (BL-21) were grown to an optical density at 600 nm of 0.7 in Luria-Bertani medium, induced at 37 °C with 0.1 mM isopropyl-1-thio-D-galactopyranoside, and cultivated for 2 h. Bacteria were then pelleted, resuspended in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl (pH 7.4), 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine), and sonicated. Insoluble material was removed by centrifugation. 30 µl of glutathione-Sepharose 4b beads (Amersham Biosciences) were added to the cleared lysate, incubated for 30 min at 4 °C, and washed three times with NETN buffer. GST pull-down assays were performed as described previously (28). Quantification was performed using the QuantityOne\textsuperscript{®} software (Bio-Rad). Binding affinity was determined as the ratio of bound protein and applied GST protein.

Kinase Assays—For kinase assays using fully purified components, active forms of the kinases (cyclin B1/Cdk1, Plk1, GSK3β, or CK2) and purified substrates (GST-NIPA WT or NIPA mutants, GST-Cdc25c, histone H1, Tau-1, and casein) were transferred into the kinase reactions, which contained 80 mM Hepes, pH 7.4, 10 mM MgCl\textsubscript{2}, 50 µM ATP, 1 µCi of [γ-32P]ATP (Amersham Biosciences), and 1 mM dithiothreitol. The kinase reaction was carried out at 30 °C for 10 min.

For kinase assays using depleted HeLa extracts, HeLa cells were lysed in lysis buffer (10 mM Tris-HCl (pH 7.5), 130 mM NaCl, 5 mM EDTA, 1% Triton X-100, 20 mM sodium phosphate (pH 7.5), 10 mM sodium PP\textsubscript{i}, (pH 7.0), 50 mM NaF, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of phenanthroline, aprotinin, leupeptin, and pepstatin), and immunodepletion was carried out using 4 µg of the indicated antibodies. Thereafter, 10 µl of the respective lysates were transferred to the kinase reactions containing GST or GST-NIPA as a substrate. The kinase reaction was carried out in a volume of 40 µl using the experimental conditions detailed above.

Xenopus Extracts—Xenopus CSF egg extracts were prepared essentially as described previously (29). CSF release was induced by adding 600 µM CaCl\textsubscript{2} to the extract. DNA and spindle morphology were examined as described previously (30).

RESULTS

Identification of the Protein Interaction Region of NIPA—To define the region of NIPA, which is required for binding to its substrate cyclin B1, various deletion mutants of NIPA were tested for their ability to form a complex with cyclin B1. GST fusion proteins of the respective deletions were engineered, and binding to Myc-tagged human cyclin B1 was assayed thereafter. This pull-down strategy was applied to circumvent any interference due to variations in the subcellular distribution of the NIPA deletions. These binding studies could delineate the region minimally required for binding to a C-terminal 50-aminooacid segment located between amino acids 352 and 402 of NIPA (Fig. 1 and supplemental Fig. 1). This region contains Ser-354, a residue previously shown to be phosphorylated in downstream, was not sufficient to mediate binding to NIPA as shown by the lack of binding of the NIPA AA327–377 mutant (Fig. 1). In addition, a new binding determinant was expected to be located between amino acids 378 and 402. Surprisingly, this determinant could be located to amino acids 395–402, a sequence that contains the previously described nuclear localization signal of NIPA (Fig. 1) (8). Since proteasomal degradation of cyclin B1 by the SCF\textsuperscript{NIPA} complex is determined to occur exclusively in the nucleus, it is tempting to speculate that...
the necessity of an intact nuclear localization signal for the binding of NIPA to cyclin B1 has evolved to ensure this nuclear restriction. Further conserved motifs, in particular a canonical protein interaction motif, were not detectable in this region. On the basis of the described features of the substrate interaction region, we suggest placing NIPA in the FBXO group of F-box proteins according to the recent nomenclature guidelines (7). 

Cyclin B1/Cdk1 Phosphorylates NIPA in Vitro—Cyclin B1/Cdk1 can control its own regulators such as Cdc25, Wee1, and the APC/C. We thus went on to investigate whether cyclin B1/Cdk1 may serve as a kinase for NIPA. First, we applied an in vitro kinase assay using purified cyclin B1/Cdk1 and GST/H18528NIPA to investigate whether cyclin B1/Cdk1 can phosphorylate NIPA in a direct manner. As depicted in Fig. 2A, cyclin B1/Cdk1 was able to specifically confer $^{32}$P to NIPA (lane 3). We further wished to investigate whether the substrate interaction region of NIPA detailed above serves as a sufficient substrate for this phosphorylation event. Fig. 2A, lane 6, demonstrates that phosphorylation of NIPA by cyclin B1/Cdk1 indeed occurs within the protein interaction region. Specificity was ascertained using GST and different deletion mutants of NIPA lacking the substrate binding region (lanes 2, 5, and 7). Of note, no phosphorylation of NIPA was observed when using active cyclin A/Cdk2 in this assay (data not shown).

Next, we searched for the relevant phosphoacceptor sites within the NIPA substrate binding region. Since we could previously demonstrate that serine residue 354 is involved in $G_2$ phase-specific phosphorylation of NIPA in vivo (9), we tested a NIPA Ser-354 point mutant (NIPA S354A) for phosphorylation by cyclin B1/Cdk1 using the in vitro setting described for Fig. 2A. Fig. 2B, lane 4, demonstrates that mutation of Ser-354 to alanine caused only minor decrease of cyclin B1/Cdk1-specific phosphorylation of NIPA, thus suggesting the presence of other phosphoacceptor sites. Since Ser-354 is not located in a consens phosphate site for Cdk1, we reasoned that Cdk1 phosphoacceptor sites present in the protein binding region may be involved. We identified three putative Cdk phosphorylation sites located on serine residues 359, 370, and 395 that resemble the core Cdk consensus site ((S/T)P) (Fig. 2C). All of these sites are conserved in human and mouse, whereas only Ser-359 and Ser-395 are conserved from human to Xenopus (Fig. 2C). Of note, only Ser-395 resembles the more complex Cdk1 consensus sequence (S/T)P(X/K/R), where X is any amino acid (31). We went on to mutate these residues to alanine and investigated these mutants as to their impact on cyclin B1/Cdk1-dependent phosphorylation of NIPA in vitro. As shown in Fig. 2B, mutation of serine 395 impaired cyclin B1/Cdk1-dependent phosphorylation of NIPA to a clearly higher extent than NIPA S354A (lane 5), whereas NIPA S370A and NIPA Ser-359 were phosphorylated to an extent similar to NIPA WT (lanes 7 and 8). Notably, a NIPA S354A,S359A double mutant (lane 6) incorporated similar amounts of $^{32}$P as NIPA WT, thus suggesting that the minor reduction in phosphorylation of the NIPA S354A mutant is likely not cyclin B1/Cdk1-specific.
Thus, Ser-395 serves as a phosphoacceptor site for cyclin B1/Cdk1 in vitro.

Next, we sought to investigate other G2/M kinases with regard to their ability to phosphorylate NIPA within the protein binding region. We performed consensus sequence analysis of this region and found Ser-354 within a consensus sequence for glycogen synthase kinase 3β (GSK3β, SXXXS) and Ser-359 within a consensus sequence for casein kinase 2 (CK2, SXX). In addition, we chose Plk1 as a further candidate, given the pivotal role of this kinase in regulating mitotic entry. The candidate kinases were subjected to an in vitro kinase assay together with GST/NIPA or known substrates of the respective kinases, and incorporation of 32P was analyzed thereafter. As shown in Fig. 2D, none of the tested kinases was able to specifically phosphorylate NIPA in vitro. Thus, GSK3β, CK2, and Plk1 are most likely not relevant kinases of the NIPA protein binding region. Of note, NIPA contains a consensus phosphorylation site for the Akt kinase (RXXR/S/T) outside of the protein binding region at serine residue 407. Some recent evidence suggests a role for Akt in regulating the mitotic and meiotic G2 to M transition by inhibiting the Wee1 and Myt1 kinases (32, 33). We therefore mutated Ser-407 to alanine and analyzed the shift in electrophoretic mobility in G2/M-arrested cells. However, this mutation was not able to affect phosphorylation of NIPA (supplemental Fig. 2). Thus, although several other candidate kinases of NIPA were investigated in this study, we were not able to identify relevant kinases other than cyclin B1/Cdk1.

Cyclin B1/Cdk1-dependent Phosphorylation of NIPA by Mitotic and Meiotic Cell Extracts—To further substantiate the role of cyclin B1/Cdk1 in phosphorylating NIPA in mitosis, we tested whether mitotic HeLa cell extracts depleted of cyclin B1 or cyclin A were able to phosphorylate NIPA. We found that extracts precleared with an anti-cyclin B1 antibody had a strongly reduced capability to phosphorylate NIPA (Fig. 3A). Depletion with an anti-cyclin A antibody or preimmune serum did not affect the phosphorylation reaction (Fig. 3A). Only mitotic extracts were able to phosphorylate NIPA, whereas interphase extracts did not possess any kinase activity toward NIPA, consistent with the recently described onset of NIPA phosphorylation in late G2 phase and persistence throughout mitosis (Fig. 3A) (9). Fig. 3B demonstrates effective depletion of both cyclin A and cyclin B1 in the respective extracts.

Next, we investigated the presence of kinase activity toward NIPA in Xenopus oocytes, using either metaphase-arrested CSF extracts or Ca2+-stimulated G1 extracts. This model allows a...
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FIGURE 3. Cyclin B1/Cdk1-dependent phosphorylation of NIPA by mitotic and meiotic cell extracts. A, depletion of cyclin B1 from HeLa mitotic extracts impairs NIPA phosphorylation. GST-NIPA was incubated with either interphase (interph.) HeLa extract or mitotic HeLa extracts treated with preimmune serum (PI), anti-cyclin B1 antibodies, or anti-cyclin A antibodies in the presence of [γ-32P]ATP. To control specificity, GST was incubated with a mitotic extract treated with preimmune serum. B, depleted mitotic HeLa extracts were immunoblotted (IB) with the indicated antibodies. C, CSF-arrested Xenopus oocyte extracts phosphorylate NIPA. 32P-labeled in vitro translated NIPA was incubated with either CSF-arrested Xenopus egg extracts or extracts treated with calcium to induce CSF release. Samples were taken at the indicated time points. D, DNA morphology was observed to follow exit from CSF arrest.

Phosphorylation of NIPA in Mitosis Involves Serine Residues 359 and 395 in Vivo—To further investigate the role of cyclin B1/Cdk1 in phosphorylating NIPA in vivo, we went on to examine the NIPA point mutants described above (NIPA-354A, -359A, -370A, -395A) as to their impact on the cell cycle-dependent phosphorylation of NIPA at the G2/M transition. To this end, HeLa cell lines with stable expression of the respective constructs of NIPA were generated. These lines were synchronized at prometaphase, and phosphorylation was analyzed thereafter with regard to the shift of NIPA in electrophoretic mobility. As described previously, mutation of serine 354 to alanine largely abolished phosphorylation of NIPA at G2/M (Fig. 4A, upper panel, lanes 3 and 4). Surprisingly, both the S359A and the S395A mutants were significantly less phosphorylated at G2/M when compared with NIPA WT (Fig. 4A, upper panel, lanes 5, 6, 9, and 10). Although the decrease in phosphorylation of S359A was similar to S354A, phosphorylation of the S395A mutant as determined by electrophoretic mobility was somewhat stronger than S354A yet clearly inferior to NIPA WT. The NIPA S370A mutant displayed a wild-type phenotype (Fig. 4A, upper panel, lanes 7 and 8). A similar synchrony of the different mutants at prometaphase was ascertained by DNA content analysis (Fig. 4B). To further analyze the impact of the described mutations on the G2/M-specific phosphorylation of NIPA, we generated double mutants (NIPA S354A,S359A, NIPA S354A,S395A, NIPA S359A,S395A) and a triple mutant (NIPA S354A,S359A,S395A) thereof. Again, stable HeLa lines were generated, and the electrophoretic shift of each mutant was analyzed in G2/M-arrested cells. Although mutation of 2 residues synergistically reduced phosphorylation when compared with the single mutants of NIPA (Fig. 4A, upper panel, lanes 11–16), only the triple mutant demonstrated a near to complete loss of phosphorylation (Fig. 4A, upper panel, lanes 17 and 18). These data suggest the presence of at least 3 distinct serine residues of NIPA that are involved in the phosphorylation process in late G2 and mitosis and demonstrate that phosphorylation of the cyclin B1/Cdk1 phosphorylation site (Ser-395) is an integral part of this process. We next investigated the functional consequence of NIPA phosphorylation at Ser-359 and Ser-395. G2/M-specific phosphorylation of NIPA at Ser-354 has previously been shown to abolish binding to Skp1, thus inactivating the SCFNIPA complex (9). We therefore went on to investigate the ability of the various NIPA single, double, and triple mutants to bind to Skp1 in G2/M-arrested cells using the stably transfected HeLa cell lines described above. To this end, immunoprecipitations of the FLAG-tagged NIPA mutants were performed, and binding to endogenous Skp1 was analyzed. In these binding experiments, both NIPA Ser-359 and NIPA Ser-395 maintained binding to Skp1 in G2/M cells, whereas the binding of NIPA WT and Ser-370 was markedly reduced (Fig. 4A, lanes 1, 2, and 5–10, and 4C). NIPA S354A retained binding to Skp1, similar to NIPA S359A (Fig. 4A, lanes 3 and 4, and 4C). Similarly, the double and triple mutants of NIPA retained binding to Skp1 at G2/M (Fig. 4C, lanes 11–18). Equivalent results were

very distinguished analysis of cyclin B1/Cdk1 kinase activity since metaphase cells contain high cyclin B1/Cdk1 activity due to the Xerpl-mediated arrest just prior to APC-dependent cyclin B1 destruction and the prompt inactivation of cyclin B1/Cdk1 as cells are released from this arrest subsequent to Ca2+ stimulation (34, 35). Using this experimental approach, we found substantial and rapid phosphorylation of NIPA using CSF-arrested extracts, whereas no phosphorylation of NIPA was detectable when using the Ca2+ stimulated extracts (Fig. 3C). Microscopic examination of the extract revealed decondensed chromatin upon calcium addition, confirming that the extracts had entered interphase (Fig. 3D). This finding further underlines a function of cyclin B1/Cdk1 in phosphorylating NIPA in mitosis.

Phosphorylation of NIPA in Mitosis Involves Serine Residues 359 and 395 in Vivo—To further investigate the role of cyclin B1/Cdk1 in phosphorylating NIPA in vivo, we went on to examine the NIPA point mutants described above (NIPA-354A, -359A, -370A, -395A) as to their impact on the cell cycle-dependent phosphorylation of NIPA at the G2/M transition. To this end, HeLa cell lines with stable expression of the respective constructs of NIPA were generated. These lines were synchronized at prometaphase, and phosphorylation was analyzed thereafter with regard to the shift of NIPA in electrophoretic mobility. As described previously, mutation of serine 354 to alanine largely abolished phosphorylation of NIPA at G2/M (Fig. 4A, upper panel, lanes 3 and 4). Surprisingly, both the S359A and the S395A mutants were significantly less phosphorylated at G2/M when compared with NIPA WT (Fig. 4A, upper panel, lanes 5, 6, 9, and 10). Although the decrease in phosphorylation of S359A was similar to S354A, phosphorylation of the S395A mutant as determined by electrophoretic mobility was somewhat stronger than S354A yet clearly inferior to NIPA WT. The NIPA S370A mutant displayed a wild-type phenotype (Fig. 4A, upper panel, lanes 7 and 8). A similar synchrony of the different mutants at prometaphase was ascertained by DNA content analysis (Fig. 4B). To further analyze the impact of the described mutations on the G2/M-specific phosphorylation of NIPA, we generated double mutants (NIPA S354A,S359A, NIPA S354A,S395A, NIPA S359A,S395A) and a triple mutant (NIPA S354A,S359A,S395A) thereof. Again, stable HeLa lines were generated, and the electrophoretic shift of each mutant was analyzed in G2/M-arrested cells. Although mutation of 2 residues synergistically reduced phosphorylation when compared with the single mutants of NIPA (Fig. 4A, upper panel, lanes 11–16), only the triple mutant demonstrated a near to complete loss of phosphorylation (Fig. 4A, upper panel, lanes 17 and 18). These data suggest the presence of at least 3 distinct serine residues of NIPA that are involved in the phosphorylation process in late G2 and mitosis and demonstrate that phosphorylation of the cyclin B1/Cdk1 phosphorylation site (Ser-395) is an integral part of this process. We next investigated the functional consequence of NIPA phosphorylation at Ser-359 and Ser-395. G2/M-specific phosphorylation of NIPA at Ser-354 has previously been shown to abolish binding to Skp1, thus inactivating the SCFNIPA complex (9). We therefore went on to investigate the ability of the various NIPA single, double, and triple mutants to bind to Skp1 in G2/M-arrested cells using the stably transfected HeLa cell lines described above. To this end, immunoprecipitations of the FLAG-tagged NIPA mutants were performed, and binding to endogenous Skp1 was analyzed. In these binding experiments, both NIPA Ser-359 and NIPA Ser-395 maintained binding to Skp1 in G2/M cells, whereas the binding of NIPA WT and Ser-370 was markedly reduced (Fig. 4A, lanes 1, 2, and 5–10, and 4C). NIPA S354A retained binding to Skp1, similar to NIPA S359A (Fig. 4A, lanes 3 and 4, and 4C). Similarly, the double and triple mutants of NIPA retained binding to Skp1 at G2/M (Fig. 4C, lanes 11–18). Equivalent results were
obtained for the binding of the NIPA mutants to Cul1, a further member of the core SCF complex (Fig. 4A). Thus, in addition to Ser-354, both residues Ser-359 and Ser-395 appear to be necessary for the efficient dissociation of NIPA from Skp1 at G2/M. The functional consequence of an assembled SCFNIPA complex in mitosis is continued degradation of cyclin B1. We thus investigated the levels of cyclin B1 in mitotic extracts of the stable cell lines described above. Our results show that the physiological mitotic increase of cyclin B1 was markedly reduced in cells expressing NIPA S359A and NIPA S395A, similar to the control, NIPA S354A. Both NIPA WT and NIPA S370A retained the physiological mitotic increase of cyclin B1 (Fig. 4A, lanes 1–10, and 4D). The double and triple mutants of NIPA demonstrated a similar phenotype as the respective single mutants (Fig. 4A, lanes 11–18, and 4D). These data suggest that phosphorylation of Ser-354, -359, and -395 is required to efficiently inactivate the SCFNIPA complex at G2/M.

To rule out the possibility that mutation of Ser-359 and Ser-395 interfered with phosphorylation of Ser-354, thus undermining the role of Ser-359 and Ser-395 as independent phosphorylation sites, we investigated phosphorylation of these mutants when coexpressed with the oncogenic fusion protein NPM-ALK (36). Overexpression of NPM-ALK has previously been shown to activate a Ser/Thr kinase that phosphorylates NIPA at Ser-354 (8). To this end, we coexpressed NPM-ALK with NIPA WT or either of the NIPA-S354A, NIPA-S359A, or NIPA-395A mutants and subsequently analyzed NIPA with regard to electrophoretic mobility. Although mutation of Ser-354 completely abolished the NPM-ALK induced phosphorylation of NIPA, NIPA-Ser-359 and -Ser-395 were phosphorylated like NIPA WT, thus excluding the possibility that phosphorylation of Ser-354 is perturbed by mutating Ser-359 or Ser-395 (Fig. 4E). The Ser/Thr kinase activated by NPM-ALK may be a potential candidate for NIPA phosphorylation at

FIGURE 4. Phosphorylation of NIPA in mitosis involves serine residues 359 and 395 in vivo. A, HeLa cells were stably transfected with the indicated constructs of NIPA and were subsequently synchronized at prometaphase using a sequential thymidine/nocodazole treatment or left unsynchronized. Thereafter, FLAG-tagged proteins were immunoprecipitated (IP), and both the bound protein fraction (upper three panels) and the respective cell lysates (lower three panels) were subjected to Western blot analysis using the indicated antibodies. Phosphorylation was analyzed with regard to the shift of NIPA in electrophoretic mobility. IB, immunoblot. B, cell cycle profiles of asynchronous or synchronized cells used in A as determined by flow cytometry. C, quantification of Skp1 in the bound protein fraction shown in A. asynchr., asynchronous. D, quantification of cyclin B1 in the cell lysate shown in A. E, NIPA WT or the indicated point mutants of NIPA were cotransfected with NPM-ALK or empty vector 293T cells. Whole cell extracts were prepared thereafter, and immunoblot analysis was performed using the indicated antibodies.


G2/M under physiological conditions. Thus, this finding may further suggest the presence of distinct kinases for Ser-354 and Ser-359.

**DISCUSSION**

In this report, we have further characterized the previously identified F-box-containing protein NIPA with regard to the site of interaction with its substrate cyclin B1 and the underlying mechanism of its G2/M-specific phosphorylation. We define the protein binding region of NIPA as a C-terminal stretch spanning from amino acids 352–402. This location of the substrate binding determinant is typical for F-box proteins and present among most members of this protein family (4).

Furthermore, this binding region contains three G2/M-specific phosphorylation sites of NIPA: the previously described Ser-354 and 2 novel residues, Ser-359 and Ser-395, which will be discussed in more detail below. The presence of these sites within the substrate binding region is in line with the recently described observation that the G2/M phase-specific phosphorylation of NIPA not only abrogated binding to Skp1, thereby inactivating the SCF<sub>NIPA</sub> complex, but also terminated the binding of NIPA to cyclin B1 (9). This constellation underlines the pivotal role of the cell cycle-dependent phosphorylation of NIPA in regulating its function and may hint at a role for this putative kinase(s) for Ser-354 and Ser-359.

The present study investigated the effect of the various point mutations on the G2/M-specific shift of NIPA in stably transfected HeLa cell lines. We found that this system detects alterations of the electrophoretic mobility far more differentiated than Cos1 cells, which we utilized to identify Ser-354 as a major phosphorylation site in our previous study (9). Although mutation of Ser-354 is sufficient to abolish the G2/M-specific shift of NIPA in Cos1 cells, the use of HeLa cells enabled us to find residual phosphorylation of the NIPA S354A mutant at G2/M, which eventually contributed to identify Ser-359 and Ser-395 as further functionally relevant phosphorylation sites. These findings suggest that at least three distinct phosphorylation sites are involved in the process that eventually inactivates the SCF<sup>NIPA</sup> in late G2 phase and mitosis. Although cyclin B1/Cdk1 is identified as the kinase responsible for phosphorylation of NIPA at Ser-395, the kinase(s) for Ser-354 and Ser-359 remain unknown despite the effort described in this study.

Given that initial phosphorylation of NIPA in late G2 inactivates the SCF<sup>NIPA</sup>, thus allowing for cyclin B1 expression (9), the process of NIPA phosphorylation at G2/M is likely to be sequential. Moreover, it appears conceivable that this process involves several distinct kinases. This notion is supported by our observation that overexpression of NPM-ALK only associates with phosphorylation of Ser-354, whereas Ser-359 or Ser-395 is not phosphorylated under this condition.

NPM-ALK has previously been shown to activate a so far unknown Ser/Thr kinase that phosphorylates NIPA (8). This Ser/Thr kinase may therefore be a candidate for NIPA phosphorylation at G2/M under physiological conditions. Thus, phosphorylation of Ser-354 and Ser-359 may require distinct kinases, potentially in an interdependent manner. The identification of these kinases will remain a subject of future investigation. In summary, we suggest a model where initial phosphorylation at Ser-354 and Ser-359 dissociates the SCF<sup>NIPA</sup> complex to initiate its inactivation and cyclin B1/Cdk1 enhances phosphorylation thereafter to ensure its own activity (Fig. 5).
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