NIPP1-mediated Interaction of Protein Phosphatase-1 with CDC5L, a Regulator of Pre-mRNA Splicing and Mitotic Entry*

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An Boudrez, Monique Beullens‡, Peter Groenen§, Aleyde Van Eynde‡, Veerle Vultaekte‡, Izabela Jagiello, Michael Murray¶, Adrian R. Krainer†, Willy Stalmans, and Mathieu Bollen**

From Afdeling Biochemie and §Center for Human Genetics (VIB), Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium and ¶Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

NIPP1 is a regulatory subunit of a species of protein phosphatase-1 (PP1) that co-localizes with splicing factors in nuclear speckles. We report that the N-terminal third of NIPP1 largely consists of a Forkhead-associated (FHA) protein interaction domain, a known phosphopeptide interaction module. A yeast two-hybrid screening revealed an interaction between this domain and a human homolog (CDC5L) of the fission yeast protein cdc5, which is required for G2/M progression and pre-mRNA splicing. CDC5L and NIPP1 co-localized in nuclear speckles in COS-1 cells. Furthermore, an interaction between CDC5L, NIPP1, and PP1 in rat liver nuclear extracts could be demonstrated by co-immunoprecipitation and/or co-purification experiments. The binding of the FHA domain of NIPP1 to CDC5L was dependent on the phosphorylation of CDC5L, e.g. by cyclin E-Cdk2. When expressed in COS-1 or HeLa cells, the FHA domain of NIPP1 did not affect the number of cells in the G2/M transition. However, the FHA domain blocked β-globin pre-mRNA splicing in nuclear extracts. A mutation in the FHA domain that abolished its interaction with CDC5L also canceled its anti-splicing effects. We suggest that NIPP1 either targets CDC5L or an associated protein for dephosphorylation by PP1 or serves as an anchor for both PP1 and CDC5L.

Type 1 protein phosphatases (PP1)¹ belong to the PPP family of Ser/Thr protein phosphatases and regulate diverse cellular processes such as transcription, pre-mRNA splicing, intracellular transport, and metabolism (1–5). They consist of a single catalytic subunit (PP1c) and one or two regulatory subunits. The regulatory subunits act as substrate specifiers and anchor the holoenzymes in specific cell compartments in close vicinity to their substrates. In addition, the regulatory subunits mediate the control of the holoenzymes by hormones and growth factors through interaction with allosteric effectors or through phosphorylation by specific protein kinases. It has been estimated that mammalian cells contain tens of different regulatory proteins of PP1 (4). Altogether about 20 of these have already been characterized and cloned, including the glycogen-binding G-subunits, the myosin-binding M-subunits, the cytosolic regulator inhibitor-1, and the nuclear RNA-binding protein NIPP1 (1–3). Recent investigations have revealed that these regulatory proteins have multiple points of interaction with PP1C, including a common phosphatase binding motif with the consensus sequence RVXF (5–10). In addition, most regulatory subunits contain domains that mediate the binding to substrates (e.g. myosin for the M-subunits) and/or a subcellular structure (e.g. glycogen for the G-subunits) to which the substrates are bound.

In nuclear extracts, NIPP1 (39 kDa) is present as an inactive complex with PP1C, termed PP1NIPP1 (11). This heterodimer can be activated by phosphorylation of up to 4 Ser/Thr residues in the central domain of NIPP1 by protein kinases A and CK2 (12), which disrupts its interaction with PP1C via the RVXF motif without dissociation of the two polypeptides (10, 12). The C terminus of NIPP1 binds in vitro to AU-rich RNA sequences (13–15). Since the binding of NIPP1 to RNA and PP1C are not mutually exclusive, we have proposed that NIPP1 may function as an RNA-targeting subunit of PP1 (15). NIPP1 also co-localizes with splicing factors in the nuclear speckles, which largely correspond to the “interchromatin granule clusters” and represent storage or recycling sites for pre-mRNA splicing factors (16).

Although the N-terminal third of NIPP1 is not required for the binding of PP1C or RNA, it represents by far the most conserved fragment of NIPP1, suggesting an essential role for this domain. We show here that the N-terminal third of NIPP1 largely consists of a “Forkhead-associated” (FHA) domain that is present in a large variety of mostly nuclear proteins and preferentially binds to specific phosphopeptides (17–21). The FHA domain of NIPP1 was found to interact with a human homolog (CDC5L) of Schizosaccharomyces pombe cdc5, a regulator of pre-mRNA splicing (22, 23), and this interaction was dependent on the phosphorylation of CDC5L, e.g. by cyclin E-Cdk2. Combined with observations that the FHA domain of NIPP1 blocked pre-mRNA splicing in nuclear extracts, these data provide firm evidence for a regulatory role of PP1NIPP1 in nuclear RNA processing.

EXPERIMENTAL PROCEDURES

Materials—PP1C and PP2A were prepared from rabbit skeletal muscle (12). Recombinant bovine NIPP1 (12) and human cyclin E-Cdk2

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(24) were produced by baculovirus expression in High Five™ cells (Invitrogen). Rabbit polyclonal antibodies against synthetic peptides comprising the C termini of CDC5L (YADLLEKETLKSFK) and NIPP1 (PGKPTPLSSL), coupled to keyhole limpet hemocyanin, were affinity-purified on bovine serum albumin-coupled peptides linked to maltose-binding protein. Polyclonal antibodies against a peptide (RTPLRDKLNNPFGD) from the central domain of CDC5L were prepared as described previously (26). Goat anti-rabbit and anti-mouse secondary antibodies were obtained from Dako. Texas Red-linked sheep anti-mouse secondary antibodies and CNBr-activated Sepharose 4B were delivered by Amersham Pharmacia Biotech. Monoclonal antibodies (HA) antibodies (12CA5), restituted CHO-K1 cells expressing a keyhole limpet hemocyanin tag have been described before (26). The sequence of all DNA constructs was verified by cycle sequencing on an ALFII sequencer (Amersham Pharmacia Biotech). Oligonucleotide primers for polymerase chain reaction and Cy5-labeled fluoroprimers were delivered by Eurogentec.

Preparation of Constructs and Recombinant Proteins—Polyhistidine-tagged polypeptides (NIPP11–142, NIPP1225–331, and CDC5L260–606) were produced in BL21(DE3)pLysS cells by transformation with the pET16b plasmid. The alanine mutation of residues 68–71 in NIPP1 was introduced using the QuickChange site-directed mutagenesis kit (Stratagene). The proteins were purified on Ni2+–PDC columns (Affi-land). A construct encoding NIPP1 (C-terminally fused to the enhanced green fluorescent protein (EGFP) was made by cloning the coding sequence of bovine NIPP1 into the Xhol/SacII sites of pEGFP-N1 (Clontech). GST fusions of CDC5L (272–606), NIPP1 (1–142), and Pavo DNA-polymerase were purified from Roche Diagnostics. Oligonucleotide primers for polymerase chain reaction and Cy5-labeled fluoroprimers were delivered by Eurogentec.

For GST pull-down assays (Fig. 6), polyhistidine-tagged CDC5L260–606 was incubated overnight with phosphorylation buffer (see above) in the presence of 0.3 mM ATP and 6 mM magnesium acetate, with or without cyclin E-Cdk2 (50 milliunits/ml). The phosphorylation reaction was arrested by the addition of 32 mM EDTA. GST and the GST-fusions of the FHA domain (NIPP11–142) or the FHA mutant were incubated for 30 min at 4 °C with glutathione-agarose beads (Sigma) that had been preblocked with TBS plus bovine serum albumin (1 mg/ml), 0.5% Triton X-100, and 1 mM dithiothreitol and then equilibrated with TBS plus 1 mM dithiothreitol. After washing with TBS plus 1 mM dithiothreitol, the glutathione-agarose beads were incubated with (or without phosphorylated) CDC5L260–606 for 1 h at 4 °C. The beads were washed once with TBS, 1 mM dithiothreitol, 0.1% Triton X-100 and twice with TBS plus 1 mM dithiothreitol. The associated proteins were separated by 10% Tricine-SDS-polyacrylamide gel electrophoresis and visualized with antibodies against a central synthetic fragment of CDC5L. The same GST pull-down procedure was also used for the analysis of the association between full-length NIPP1, or an in vitro reconstituted complex of NIPP1 and PPI1α, and Cdk2-phosphorylated GST-CDC5L260–606. The co-precipitation of PPI1α-GFP, and CDC5L was evaluated by the assay of phosphorylase phosphatase activity after a preincubation with trypsin, which liberates PPI1α (12). Finally, the GST pull-down assay was also used to analyze the phosphorylation dependence of the interaction of CDC5L in nuclear extracts to GST-NIPP11–142. All constructs were produced in BL21(DE3)pLysS cells by transformation with the pET16b plasmid. The alanine mutation of residues 68–71 in NIPP1 was introduced using the QuickChange site-directed mutagenesis kit (Stratagene). The proteins were purified on Ni2+–PDC columns (Affi-land). A construct encoding NIPP1 (C-terminally fused to the enhanced green fluorescent protein (EGFP) was made by cloning the coding sequence of bovine NIPP1 into the Xhol/SacII sites of pEGFP-N1 (Clontech). GST fusions of CDC5L (272–606), NIPP1 (1–142), and Pavo DNA-polymerase were purified from Roche Diagnostics. Oligonucleotide primers for polymerase chain reaction and Cy5-labeled fluoroprimers were delivered by Eurogentec.

RESULTS

FHA Families—A ProfileScan analysis² showed that the N terminus of NIPP1 contains a previously unrecognized FHA domain (Fig. 1). Sequence alignments of various FHA-containing proteins revealed that there actually exist at least two families of FHA domains. NIPP1 contained an FHA domain highly similar to that found in the putative proline isomerase pinA (GenBank™ accession no. U78757) and in kanadaptin (GenBank™ accession no. AF035526), a protein that binds to a Cl−/HCO3− exchanger (31). Surprisingly, the FHA domain of kanadaptin was mapped upstream of the suggested open reading frame, indicating that kanadaptin may be a larger protein than previously predicted.

2 ProfileScan is available on the World Wide Web.
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A Two-hybrid assay

B Interacting domain of CDC5L

The NIPP1–CDC5L Interaction Is Controlled by Phosphorylation—While the above data show that NIPP1 and CDC5L are part of the same complex in the nucleus, they do not prove a direct interaction between both components. A direct interaction between recombinant NIPP1 and CDC5L could not be demonstrated, however, by co-precipitation (see below) and far Western analysis (not shown). In view of recent findings...
that FHA domains represent phosphopeptide-interacting motifs (20, 21), we explored the role of the phosphorylation of CDC5L on its interaction with the FHA domain of NIPP1. CDC5L contains numerous consensus sites ((S/T)P (R/K)) for phosphorylation by proline-directed kinases (Fig. 2B). CDC5L was indeed an excellent substrate for in vitro phosphorylation by cyclin E-Cdk2, reaching final stoichiometries of phosphorylation of 4.3 mol of phosphate/mol of CDC5L (not shown). Moreover, following its phosphorylation by cyclin E-Cdk2, CDC5L interacted with a GST fusion of the FHA domain of the FHA domain of NIPP1, as determined by co-precipitation analysis (Fig. 6). On the other hand, phosphorylated CDC5L did not co-precipitate with a GST fusion of an FHA domain in which residues 68–71 were substituted for alanine. Collectively, these data indicate that the FHA domain of CDC5L is phosphorylated in hepatic nuclei and that this phosphorylation is required for its interaction with NIPP1. We have also explored whether Cdk2-phosphorylated CDC5L is a substrate for dephosphorylation by PP1C (Table I). Compared with the reference substrate phosphorylase a, CDC5L was dephosphorylated by PP1C with a 9-fold lower V_max and a 4-fold lower K_m. Thus, CDC5L was dephosphorylated with an efficiency (V_max/K_m) that was only 2-fold lower than that of phosphorylase a. As previously demonstrated for phosphorylase a (12), the dephosphorylation of CDC5L by PP1C was completely blocked by the addition of NIPP1. Also, NIPP1 became a less potent inhibitor of CDC5L dephosphorylation following its phosphorylation by protein kinases A plus CK2 (not illustrated). We have also found that CDC5L could be dephosphorylated by PP2A C (Table I). Compared with phosphorylase a, the dephosphorylation of CDC5L by PP2A C occurred with a 2-fold higher V_max and a 6-fold higher K_m (i.e. with a 2.7-fold lower catalytic efficiency). Overall, PP1C acted severalfold more efficiently on CDC5L than did PP2A C.

Role of the FHA Domain of NIPP1 in Pre-mRNA Splicing—Since CDC5L has been implicated in both the G_2/M transition and in pre-mRNA splicing (20, 22), we have investigated whether the FHA domain of NIPP1 perhaps also plays a role in these cellular processes. We found that the expression in COS-1 cells of HeLa cells of either full-length NIPP1 or its FHA domain (residues 1–142), C-terminally fused to the enhanced green fluorescent protein, did not affect the number of cells in the G_2/M transition, as determined by FACS analysis (not illustrated). On the other hand, the N terminus of NIPP1 blocked the splicing of β-globin pre-mRNA in HeLa cell nuclear extracts (Fig. 8). This inhibition was dependent on the concentration of the FHA domain and was complete at 10 μM. By contrast, the mutated FHA domain, which did not interact with CDC5L (Figs. 2A, 6, and 7), did not show any anti-splicing effect (Fig. 8).

DISCUSSION

FHA Domains Are Phosphopeptide-interacting Motifs—The FHA domain of the PP2C-like protein phosphatase KAPP only recognizes the phosphorylated form of a receptor-like protein kinase domain (18). Likewise, the FHA domains of the RAD53 protein kinase are selectively recruited to phosphorylated Rad9, a component of the DNA damage checkpoint (19–21). We report here that the FHA domain of NIPP1, which belongs to a different family of FHA-containing proteins (Fig. 1), also interacts with CDC5L in a phosphorylation-dependent manner. Moreover, the alanine mutation of residues 68–71 of NIPP1 abolished its interaction with CDC5L (Figs. 2A, 6, and 7). Since residues 68–71 of NIPP1 correspond to a loop region in the FHA domain of RAD53 that is essential for the binding to phosphopeptides (21), this effect of the mutation strongly indicates that the NIPP1–CDC5L interaction requires an intact FHA domain and is mediated by a phosphorylated fragment of CDC5L.

The Role of CDC5L and PP1NIPP1 in Pre-mRNA Splicing—Various observations implicate both CDC5L and PP1NIPP1 in nuclear pre-mRNA processing. First, both CDC5L (23, 33) and
NIPP1 (Ref. 16; Fig. 5) co-localize with splicing factors. Cyclin E-Cdk2, which phosphorylates CDC5L and enables its interaction with NIPP1 (Figs. 6 and 7), is also associated with splicing factors in mammalian cells (34). Second, PP1 (35) and fission yeast cdc5 (23) are required for the first step in pre-mRNA splicing. Third, immunodepletion of NIPP1 or the addition of a dominant negative NIPP1 mutant inhibited pre-mRNA splicing \[ \text{in vitro} \] (16). We report here that the FHA domain of NIPP1 also blocks splicing in nuclear extracts (Fig. 8). Since the FHA mutant did not show this anti-splicing effect, it is likely that the inhibition is mediated by the interaction of the FHA domain with CDC5L. A likely explanation for the inhibition of splicing by the FHA domain of NIPP1 is that it prevents the interaction of endogenous NIPP1 with CDC5L.

The exact role of CDC5L and PP1\textsubscript{NIPP1} in pre-mRNA splicing remains to be established. One view is that NIPP1 functions as a CDC5L-targeting subunit of PP1. In accordance with this hypothesis, we found that CDC5L can be dephosphorylated \textit{in vitro} by PP1\textsubscript{C} with an efficiency that is similar to that of the model substrate phosphorylase \( \alpha \) (Table I). However, at variance with the function of other targeting subunits of PP1, such as the glycogen-binding and myosin-binding subunits, NIPP1 did not enhance the dephosphorylation of CDC5L by the associated PP1\textsubscript{C}, but, in contrast, potently inhibited its dephosphorylation. The possibility cannot be excluded, however, that the dephosphorylation of CDC5L is affected by other components of the CDC5L-PP1\textsubscript{NIPP1} complex or is controlled by additional (covalent) modifications. Thus, we found that NIPP1 became a less potent inhibitor of CDC5L dephosphorylation following its phosphorylation by protein kinases A and CK2, similar to what we have previously described for the dephosphorylation of other substrates of PP1 (12). An alternative view

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**Fig. 5. Co-localization of NIPP1 and CDC5L in the nuclear speckles.** NIPP1 and CDC5L were expressed in COS-1 cells as EGFP-tagged or HA-tagged proteins, respectively. The cells were fixed, and the tagged proteins were visualized directly by green fluorescence microscopy (NIPP1-EGFP; A) or by immunofluorescence using Texas Red-labeled secondary antibodies (CDC5L-HA; B). C shows an overlay of both stainings. Shown are the nuclei of two representative cells. EGFP alone, when expressed in COS-1 cells, showed a uniform distribution between the nucleus and the cytoplasm and was not enriched in the nuclear speckles (not shown). The HA antibodies only showed a very weak and uniform background staining in cells that did not express HA-CDC5L (not shown).

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**Fig. 6. The interaction between recombinant CDC5L\textsubscript{260–606} and NIPP1\textsubscript{1–142} is dependent on phosphorylation.** Equivalent amounts of GST, GST-NIPP1\textsubscript{1–142}, and GST-NIPP1\textsubscript{1–142} that was alanine-mutated in residues 68–71 of the FHA domain (FHA-mutant), were bound to glutathione-agarose beads. The washed beads were incubated with CDC5L\textsubscript{260–606} or with cyclin E-Cdk2 phosphorylated CDC5L\textsubscript{260–606}. The resedimented and washed beads were supplemented with SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The retained CDC5L\textsubscript{260–606} (41 kDa) was visualized by Western analysis with polyclonal antibodies against the central domain of CDC5L.
been incubated for 10 min at 30 °C with 6 nM PP1C and 50 nM PP2AC in the absence of binding to the GST fusions of CDC5L from nuclear extracts, as determined by the same pull-down assay that was also used in Fig. 6. Lanes 5 and 6 show the absence of binding to the GST fusions of CDC5L from nuclear extracts that had been prepared without protein phosphatase inhibitors and had been incubated for 10 min at 30 °C with 6 nM PP1C and 50 nM PP2AC before addition to the GST fusions.

**TABLE I**

| Substrate                        | Protein phosphatase |
|----------------------------------|---------------------|
|                                  | PP1C               | PP2AC               |
| Phosphorylase α                  |                     |                     |
| $K_m$ (μM)                       | 11.8 ± 2.3          | 2.8 ± 0.7           |
| $V_{max}$ (units/mg)             | 12.7 ± 1.7          | 1.2 ± 0.3           |
| $V_{max}/K_m$ ratio              | 1.08                | 0.43                |
| CDC5L260−606                     | 3.0 ± 0.4           | 17.8 ± 2.4          |
| $K_m$ (μM)                       |                     |                     |
| $V_{max}$ (units/mg)             | 1.4 ± 0.1           | 2.8 ± 0.3           |
| $V_{max}/K_m$ ratio              | 0.46                | 0.15                |

is that the physiological substrate(s) of PP1NIPP1 is not (only) CDC5L but (also) CDC5L-associated protein(s). There is no information yet on CDC5L-associated proteins in higher eukaryotes, except that CDC5L is part of in vitro assembled spliceosomes (33) and is targeted to the nuclear speckles in COS-1 cells (Ref. 23, Fig. 5). However, fission yeast cdc5 (22) and its budding yeast homolog Cef1 (36) have been shown to be part of a multiprotein complex, which in the latter case was shown to bind tightly to the spliceosomes during splicing. Perhaps mammalian homolog(s) of Cef1-associated proteins represent the physiological substrate(s) of PP1NIPP1. PP1 has also been implicated in the recycling of splicing factors from sites of active splicing to the nuclear speckles, which for the most part represent storage and assembly sites for splicing factors (37).

Thus, CDC5L and PP1NIPP1 may be involved in both the splicing reaction itself and in spliceosome disassembly.

**NIPP1 Interacts with CDC5L**

NIPP1 was present in some nuclear speckles that contained little or no CDC5L (Fig. 5). Moreover, the CDC5L antibodies only precipitated a small fraction of NIPP1 (Fig. 3). Therefore, the association of NIPP1 with the speckles can only partly be accounted for by its interaction with CDC5L, and there may exist additional protein(s) in the speckles that bind to NIPP1. Alternatively, the association of CDC5L with the speckles is mediated by NIPP1. In the latter case, NIPP1 may turn out to be a targeting subunit of both PP1C and CDC5L.

The Role of CDC5L and PP1NIPP1 in the G2/M Transition—We did not find an effect of overexpressed NIPP1 in COS-1 cells and HeLa cells on the number of cells in the G2/M transition. On the other hand, fission yeast cdc5 was originally identified in a screen for “cell division cycle” mutants as a gene that is required for the G2/M transition (38). Likewise, Cef1 is essential for mitotic progression (22, 36). Mammalian cells overexpressing CDC5L have a shortened G2 and a reduced cell size, whereas cells overexpressing a carboxyl-terminal fragment of CDC5L display a slowed G2 progression (39). Some investigations have focused on the function of cdc5 as a putative transcription factor to explain its role in the G2/M transition. The N terminus of cdc5 and its homologs contains two Myb-like repeats with evenly spaced hydrophobic residues that may make up the backbone of the DNA-binding motif (40). These repeats were found to be essential for the function of Cef1 (41) and to bind preferentially to the DNA sequence CT-CAGCG (42). The central region of CDC5L, which is included in the NIPP1 interacting domain (Fig. 2B), shows similarities with the hydrophilic, proline-rich transactivation domain of some transcription factors. Moreover, a chimeric molecule consisting of N-terminally nicked CDC5L fused to the GAL4 DNA-binding domain transactivated a reporter gene in COS cells (39). Thus, CDC5L may function both as a transcription and a splicing factor. These functions are not mutually exclusive, since there is increasing evidence for a link between cell cycle and splicing (22, 34). One can speculate on the existence of a checkpoint that ensures that cells enter into mitosis only when there are no pre-mRNAs left. Alternatively, some transcripts...
encoding cell cycle regulators may be spliced in a cell cycle-de-pendent manner. The association of cyclin-dependent kinase(s) with the spliceosomes (34) is highly suggestive of cell cycle-regulated splicing. It should also be noted that CDC5L was identified as a mitotic phosphoprotein (43) that can be phospho-rylated in vitro by cyclin B-cdc2. Thus, both cyclin B-cdc2 (43) and cyclin E-cdk2 (this work) may represent CDC5L ki-nases. However, we found that CDC5L is phosphorylated in nuclear extracts from the liver (Fig. 7), which mostly consists of cells in G0, when cyclin E-cdk2 is inactive. Therefore, we suggest that there are other proline-directed protein kinases that can phosphorylate CDC5L during G0 and control its inter-action with NIPP1.

The identification of additional interacting proteins of CDC5L and NIPP1 is likely to result in a better understanding of their role in pre-mRNA splicing and cell cycle progression. Moreover, an analysis of the regulation of these proteins by phosphorylation may also lead us to the physiological sub-strates of PP1NIPPP1.

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