PINA is essential for growth and positively influences NIMA function in *Aspergillus nidulans*

James D. Joseph, Scott N. Daigle2 and Anthony R. Means1
Department of Pharmacology and Cancer Biology
Duke University Medical Center, Durham, NC 27710

Running Title: PINA is essential for *Aspergillus* proliferation

Key Words: Pin1, PINA, NIMA, *Aspergillus*, cell cycle

1Corresponding Author: Dr. Anthony R. Means
Department of Pharmacology and Cancer Biology,
Duke University Medical Center,
Box 3813,
Durham, NC 27710
USA
phone: (919) 681-6209
fax: (919) 681-7767
E-mail: means001@mc.duke.edu

2Current Address: Schering-Plough Corporation, Kenilworth, NJ 07033
ABSTRACT

The phospho-Ser/Thr directed prolyl-isomerase Pin1 was originally identified in vertebrate systems as a negative regulator of NIMA, a Ser/Thr protein kinase that regulates the G2/M transition in *Aspergillus nidulans*. Here we explore the physiological roles of the Pin1 ortholog, PINA, in *A. nidulans* and evaluate the relevance of the interaction of PINA with NIMA in this fungus. We find *pinA* to be an essential gene in *A. nidulans*. In addition, when PINA levels are reduced fifty-fold the cells grow at a reduced rate. Upon germination under conditions which repress PINA expression the cells are delayed in the interphase activation of NIMX<sup>cdc2</sup>, while they traverse the other phases of the cell cycle at a similar rate to controls. These results indicate that a marked reduction of PINA results in a lengthening of G1. Additionally, PINA repression increases the rate at which the cells enter mitosis following release from a hydroxyurea arrest without altering the sensitivity of the fungus to agents which activate the replication or DNA damage checkpoints. In contrast to predictions based on Pin1, the physical interaction between PINA and NIMA is primarily dependent upon the prolyl-isomerase domain of PINA and the C-terminal 303 amino acids of NIMA. Finally, reduction of PINA levels exacerbates the *nimA5* temperature sensitive mutant while overexpression of PINA decreases the severity of this mutation, results that are consistent with a positive genetic interaction between PINA and NIMA. Thus, while PINA is essential and positively regulates NIMA function, *A. nidulans* is most sensitive to a reduction in PINA concentration in G1 rather than in G2/M.
INTRODUCTION

Pin1 is an evolutionarily conserved member of the parvulin family of proline isomerases that selectively targets proteins containing proline preceded by phospho-Ser/Thr residues (reviewed in 1,2). The phospho-epitopes recognized by Pin1 show remarkable sequence similarity with that of the mitosis specific antibody, MPM-2, and are frequently generated by cdk2 or MAP kinase-dependent phosphorylation (3). Based on Pin1 interaction studies as well as predictions based on substrate specificity and analysis of Pin1 function in multiple eukaryotic systems, Pin1 has been implicated in numerous signaling pathways critical for cell proliferation. However, the phenotypic consequences of Pin1 deletion, depletion or inhibition vary greatly among organisms. Whereas Pin1 is essential for growth in Saccharomyces cerevisiae and Candida albicans, only subtle phenotypes are observed when Pin1 is deleted in Schizosaccharomyces pombe, and quite specific ones occur in Drosophila and mouse (4-12).

As might be predicted from its substrate specificity, Pin1 has been demonstrated to play an important role in regulating both mitosis and G1. Indeed, temperature sensitive mutants of the S. cerevisiae and C. albicans Pin1 homologue, Ess1p, arrest cells in mitosis (5,13). Furthermore, expression of Pin1 anti-sense RNA in HeLa cells has been reported to induce either a mitotic arrest (14) or an interphase arrest with high levels of apoptosis (15). However, at least in the case of S. cerevisiae, the mitotic role of Ess1p appears to result from transcriptional alterations rather than via direct interaction with critical mitotic regulatory proteins (13). Regardless, Pin1 has been demonstrated to interact with a number of signaling molecules involved in mitotic progression including cdc25, wee1, plk1 and NIMA (14,16,17). In contrast to the mitotic arrest observed when
Pin1 is depleted from HeLa cells, MEFs derived from the Pin1 null mouse display no detectible mitotic phenotype, but rather proliferate slightly more slowly than controls and are impaired in their ability to reenter the cell cycle after serum starvation (9,18). In support of a G1 role for Pin1, it is required for the proper timing of primordial germ cell proliferation due to a lengthening of G1 and has been reported to positively regulate c-jun, β-catenin and cyclinD1, all of which are involved in G1 progression (10,11,19-21).

Although Pin1 has been demonstrated to interact with and regulate a number of phospho-proteins in numerous systems, it was initially identified as an interactor and suppressor of the lethality induced by overexpression of the Aspergillus nidulans essential G2/M regulatory protein kinase, NIMA, in a yeast two-hybrid screen (14). The identification of Pin1 as a negative regulator of NIMA was supported by the observations that not only is the mitotic arrest induced by NIMA overexpression in HeLa cells abrogated by co-expression of Pin1, but overexpression of Pin1 in the absence of active NIMA arrests the cells in G2. As in HeLa cells, NIMA overexpression in Aspergillus induces a pseudomitotic state characterized by microtubule depolymerization and chromatin condensation. On the other hand, the repression of NIMA activity arrests cells in G2 (22). Thus, the two-hybrid and HeLa cell results are consistent with the notion that Pin1 could function as a negative regulator of NIMA and a NIMA-like signaling pathway in mammalian cells.

In order to understand the in vivo relevance of the interaction of NIMA with Pin1 we have evaluated the function of the A. nidulans Pin1 homologue, PINA. Similar to the results in the yeasts S. cerevisiae and C. albicans disruption of PINA in A. nidulans is lethal. However, using an inducible expression system, we find that a 50-fold reduction
of PINA expression is sufficient to allow growth, albeit at a markedly slower rate than the wild-type strain. In contrast to the mitotic arrest due to ess1ts mutants, yet similar to defects in Pin1 null cells, PINA depletion delays the timing of G1 progression. Furthermore, we identify a potential role for PINA in regulating the recovery from a replication checkpoint arrest since cells with reduced levels of PINA enter mitosis more quickly than controls following release from a hydroxyurea induced arrest without sensitizing the cells to the drug. Finally, in contrast to predictions of earlier studies in S. cerevisiae and HeLa cells, PINA genetically interacts with and is a positive regulator of NIMA kinase function in A. nidulans.

MATERIALS AND METHODS

A. nidulans and S. cerevisiae strains and culture techniques

The A. nidulans strains used in this study were GR5(pyrG89; pyroA4; wA3), SO6(pyrG89; yA2; wA2; cnxE16; nimA5; choA1; cha1); SWJ32(nimG10; pyrG89; nicA2; chaA1), SWJ193(pabaA1; wA2; nimE6; methB3), CDS46(nimT23; pyrG89; one extra copy nimA-4xHA; nicA2; chaA1), JJ31(alcA:pinA; pyroA4; wA3), JJ32(pyroA4; wA3), JJ32(alcA:pinA; nimG10), JJ33(nimG10), JJ34(alcA:pinA; yA2; wA2; cnxE16; nimA5; choA1; cha1), JJ35(yA2; wA2; cnxE16; nimA5; choA1; cha1) JJ36(alcA:pinA; wA2; nimE6). JJ37(nimT23; pyrG89; one extra copy nimA-4xHA; alcA:pinA; chaA1). A. nidulans strains were propagated in standard minimal medium containing either dextrose, glycerol or glycerol plus threonine as the carbon source and the appropriate nutritional supplements at either 30°C or 37°C unless otherwise indicated (23). Benomyl (Sigma-Aldrich) and hydroxyurea (Sigma-Aldrich) were used a final concentration of 5 µg/ml.
and 20 mM respectively. All genetic crosses were performed as described by Pontecorvo (24).

For two-hybrid interaction analysis pAD (carrying \textit{LEU2}) and pBD (carrying \textit{TRP1}) vectors containing various inserts were cotransformed into strain YRG-2 (\textit{Mat\alpha, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3 112, gal4-542, gal80-538, LYS2::UAS\textit{GAL1}–TATA\textit{GAL1}–HIS3, URA3::UAS\textit{GAL4} 17mers(x3)–TATA\textit{CYC1}–lacZ}) and plated on medium lacking leu and trp. To test for protein interaction the transformants were plated onto medium lacking leu, trp and his. Standard \textit{S. cerevisiae} transformation and culture techniques were used (25).

**Generation of \textit{A. nidulans} vectors and transformation**

pPINAdis was generated by cloning a portion of the PINA gene containing nucleotides 55 to 552 (equivalent to 55 to 499 of cDNA, generated by PCR of the genomic PINA clone) into the EcoRI and SmaI sites of pRG1 (26). The pAlcPINA vector was generated by cloning the 5’ end of the PINA gene into the SmaI site of pAL3 as a blunted HindIII/XmnI fragment. (26) All vectors were sequenced prior to use.

\textit{A. nidulans} GR5 cell were transformed as described by Lu and Means (23). Positive transformants were selected by growth on minimal medium in the absence of uracil and uridine to select for the presence of the \textit{pyr4} nutritional marker. Transformants of pPINAdis were maintained as heterokaryons by transfer of mycelia instead of spores. Southern analysis was performed on vegetatively growing mycelia as described by Rasmussen \textit{et al.} (27). Transformants of pAlcPINA were streaked three times to ensure strain purity followed by Southern analysis and western analysis to ensure homologous integration and protein expression.
**Microscopy and growth assays**

Nuclear number and staining were performed by growing spores in minimal medium on glass coverslips at a concentration of $10^4$ spores/ml. Germlings were collected at various times after germination followed immediately by fixing and staining with 4’,6-diamidino-2-phenylindole or Hoechst 33258 as described by Harris et al. (28). Chromosome mitotic indices were determined using cells germinated in liquid medium at 30°C and 200 rpm for approximately 5 hr followed by cell cycle arrest via temperature shift to 42°C or the addition of 20mM hydroxyurea for 3 hr. Cells were then released into fresh medium or 30°C and samples were fixed and stained and scored for mitotic nuclei as described by Lu and Means (23).

**Western blotting and immunoprecipitation**

Rabbit anti-PINA polyclonal antibodies were generated using keyhole limpet hemocyanin-conjugated, bacterially-expressed PINA as the antigen. The coupled protein was injected into rabbits, and antiserum was collected using standard techniques (29). Western analysis of PINA was performed using a 1:5000 dilution of the anti-PINA antisera and detected using either $^{125}$I-Protein A (Amersham) at a concentration of $10^5$ cpm/ml or HRP conjugated anti-rabbit IgG. Western analysis of phospho-Ser10 histone H3 was performed as described by DeSouza et al. (30). Western analysis of HA-NIMA was performed using the 12CA5 monoclonal antibody (Roche) at a dilution of 1:1000 followed by detection using HRP-conjugated anti-mouse IgG. MPM-2 reactive epitopes were detected using 1 µg/ml MPM2 monoclonal antibody (Upstate Cell Signaling) followed by detection via HRP-conjugated anti-mouse IgG.
HA-NIMA and PINA were immunoprecipitated from *A. nidulans* extracts prepared by grinding flash frozen germlings prepared as described by Dayton *et al.* (31). For immunoprecipitation of both PINA and NIMA 500 µg of protein was brought to a total of 500 µL with lysis buffer followed by the addition of the primary antibody (2 µl of anti-PINA and 1µg anti-HA). Following rocking for 1 hr at 4°C approximately 20 µL of protein A or G sepharose (Amersham-Pharmacia) was added and incubated for an additional hr. Following binding, the beads were washed 5 times in lysis buffer. The bound proteins were eluted with the addition of 30 µl SDS-PAGE protein loading buffer and detected by western analysis following SDS-PAGE electrophoresis and transfer to Immobilon P membrane (Millipore).

**Generation of *S. cerevisiae* vectors**

The NIMA and PINA truncations and mutations were cloned into pBD-GAL4 or pAD-GAL4 (Stratagene) respectively. Full-length NIMA was cloned into pBD-GAL4 as described in Crenshaw *et al.* (17). NIMA truncations (amino acids 1-292, 293-699, 293-396 and 397-699) were generated by PCR and subcloned into pBD-GAL4 as SmaI/SalI fragments. Full-length PINA in pAD-GAL4 was originally generated by Crenshaw *et al.* (17). PINA point mutants (W33A and C126A) were generated in pAD-GAL4 using the mega-primer mutagenesis technique (32). PINA truncations, WW-domain (amino acids 1-68) and PI-domain (amino acids 56-176) were generated by PCR and subcloned as EcoRI/XhoI fragments into pAD-GAL4. All vectors were sequenced prior to use.

**NIMA and NIMX kinase assays**
Extracts for NIMX^cdc2 histone H1 kinase were prepared and assays were performed as described previously (33). ^32P labeled phosphate incorporation of SDS-PAGE separated reactions was quantified using a Molecular Dynamics PhosphorImager.

Full-length, bacterially expressed NIMA used for the \textit{in vitro} kinase assays was generated by first cloning the NIMA cDNA into pET30 expression vector (Novagen) as an Ncol/SalI fragment. NIMA was expressed in freshly transformed BLR(DE3)pLysS competent cells (Novagen) grown at 37\(^\circ\)C to an O.D.\textsubscript{600nm} of 0.6 at which point the cultures were shifted to room temperature and protein expression induced with the addition of 0.4 mM IPTG for 3 hr. Following centrifugation the bacteria was resuspended in lysis buffer (25mM Tris HCl pH 8.0, 1 mM DTT, 5 mM EDTA, 1 mg/ml Pefabloc) and lysed by sonication. Following sonication, Triton-X100 was added to a final concentration of 0.5% and incubated on ice for 30 min. The extract was then clarified by centrifugation and incubated with nickel nitrilotriacetic acid-agarose (Qiagen) for 1 hr with rocking at 4\(^\circ\)C. The bound resin was then washed with 20 ml of lysis buffer plus 0.5% triton X-100, followed by 20 ml lysis buffer plus 0.5% triton X-100, 150 mM NaCl and 10 mM imidazol. The bound protein was eluted in lysis buffer containing 250 mM imidazol and stored at \(-80\)\(^\circ\)C in 40% glycerol. Recombinant PINA was expressed, purified and cleaved from the GST tag as described by Crenshaw \textit{et al.} (17).

NIMA \textit{in vitro} and IP kinase assays were performed using 100 ng of recombinant NIMA or NIMA immunoprecipitated from 500 \(\mu\)g \textit{A. nidulans} extract using either the anti-HA 12CA5 monoclonal antibody or anti-HA 3F10 conjugated sepharose. Immunoprecipitation was as described above with 2 additional washes with kinase buffer
Conditions for the kinase assay were as follows: 50 mM Tris HCl pH 8.0, 1 mM DTT, 0.1% Triton X-100, 10 mM MgCl₂, 500 µM ATP, 1 mM F-peptide (GRFRRSRRMI), 0.2 µl/reaction [γ-³²P]ATP. Reactions were performed in 30 µl (for recombinant NIMA) or 50 µl (for immunoprecipitated NIMA) volumes for 10 min and terminated by spotting 20 µl of the reaction onto p81 phosphocellulose filters followed by extensive washing in 75 mM phosphoric acid. Activities were determined followed scintillation counting of the dried filters. When applicable purified, cleaved PINA or BSA was added to the kinase and incubated for 20 min. prior to the initiation of the kinase assay.

Phosphatase assays were performed at 30°C using purified PP2A (Calbiochem) in the 50 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1% BME, and 2 mM MnCl₂ at a final concentration of approximately 0.02 unit PP2A / 50 µg NIMA. Time courses of dephosphorylation terminated either by the addition of protein loading buffer for observation of phosphorylation or the addition of 0.5 µM okadaic acid followed by kinase assay for activity measurements. Either BSA or PINA was incubated with the recombinant NIMA in assay buffer 20 min prior to the initiation of reaction.

Hydroxyurea and UV sensitivity assay

Hydroxyurea sensitivity of the AlcPinA and control strains as determined by spotting 1 µl of approximately 5 spores/ml onto glycerol or glucose minimal medium plates containing graded concentrations of hydroxyurea of up to 10 µg/ml. Growth was compared 3 days after germination at 37°C. UV sensitivity of the AlcPinA and control strains as compared by UV irradiating germlings 4 hr post germination in minimal medium plus dextrose or glycerol using a Stratalinker UV crosslinker (Stratagene) at
various intensities. Following UV irradiation the approximately 500 germlings/plate were plated on minimal medium plates containing either glucose or glycerol and 0.01% Triton X-100. Percent survival was determined after 4 days of growth at 32°C.

RESULTS

PINA is essential in *Aspergillus nidulans*

To address the *in vivo* function of PINA in *Aspergillus nidulans*, we attempted to disrupt the endogenous *pin*A gene by homologous recombination in the haploid fungus. A schematic representation of the disruption strategy is presented in Figure 1A. Despite screening over 150 integrated strains by Southern analysis we were unable to identify any strains in which the endogenous *pin*A gene was disrupted. Since *A. nidulans* normally grows in the haploid state the disruption of an essential gene would result in non-viable progeny, thus the inability to identify viable *pin*A disruptants is consistent with *pin*A being an essential gene.

To confirm that *pin*A is essential in *A. nidulans* we disrupted the gene in a heterokaryon in a manner similar to that described for *cmk*A, *cmk*B, CaM and *cna*A (27,33-35). As diagramed in Figure 1B, heterokaryon strains can be generated by the disruption of a gene following DNA synthesis or by the fusion of the parental strain with a strain containing the targeted disruption. By this method, heterokaryon strains in which essential genes are disrupted are maintained by the presence of the selectable nutritional marker in the disrupted nucleus and the presence of the wild-type targeted allele in the parental nucleus. The heterokaryon is then propagated by transfer of multinucleate mycelia. When *pin*A-targeted transformants were maintained as heterokaryons multiple
strains were identified in which *pinA* was disrupted as indicated by Southern hybridization bands at approximately 0.9 Kbp. (representing the wild-type *pinA* allele) and 3.0 Kbp. (representing the *pinA* disrupted allele), three examples of which are presented in Figure 1C. As represented in Figure 1B the sporulation of a heterokaryon strain generates two populations of spores, one representing the maternal strain which contains a wild-type allele of the targeted gene but no nutritional marker (in our case *pyr4−*/*pinA+*), while the second contains both the disrupted gene and the selectable nutritional marker (*pyr4+/pinA−*). Thus, if *pinA* is an essential gene, inoculation of the heterokaryon progeny in medium without uracil and uridine would yield no viable germlings, and in medium containing the required nutrients only the spores representing the maternal nuclei will germinate. When the *pinA* disrupted heterokaryons were grown in the absence of uracil and uridine less than one percent of the spores germinated, however, in the presence of uracil and uridine approximately 54 percent of the spores underwent multiple rounds of nuclear division and initiated polarized growth within 10 hr, the remaining spores germinated but arrested with a single nuclei and failed to initiate polarized growth (Figure 1D). The absence of the disrupted *pinA* allele in the fungus isolated following growth in the presence of uracil and uridine was confirmed by Southern analysis (data not shown). Thus, similar to its homologues in both *S. cerevisiae* and *C. albicans*, *pinA* is an essential gene in *A. nidulans*.

Reduction of PINA levels slows *A. nidulans* growth

To generate a system in which we could evaluate the physiological roles of PINA in *A. nidulans*, we created a strain in which the endogenous *pinA* gene was placed under the control of the regulable *alcA* promoter. The homologous integration of the *alcA*
promoter has proven useful for regulating the expression of numerous *A. nidulans* genes in a carbon source dependent manner (26,36,37). When grown in dextrose as the sole carbon source transcription from the *alcA* promoter is actively repressed. This repression is relieved in the presence of glycerol allowing low levels of transcriptional activity and transcription from the *alcA* promoter is induced in the presence of glycerol plus threonine. A schematic diagram representing the homologous integration of pAlcPinA into the endogenous *pinA* locus is depicted in Figure 2A. Following transformation we identified several strains by Southern analysis in which pAlcPinA had integrated into the *pinA* locus (data not shown). The multiple strains all displayed similar carbon source-dependent PINA expression, and a single strain was randomly chosen for analysis. The carbon source-dependent regulation of the AlcPinA strain in comparison to the nutritionally complemented control strain, generated by transformation of the parental GR5 strain with pAL5 vector containing no cDNA insert, (Pal5) is demonstrated in Figure 2B. When AlcPinA is grown in dextrose (MMD) PINA expression is reduced approximately 50-fold in comparison to controls. In glycerol (MMG) expression is reduced approximately 3-fold. In glycerol plus threonine (MMG+T) PINA expression is induced approximately 3-fold. The Pal5 strain displayed no variation of PINA expression in the three different carbon sources.

When germinated in the presence of dextrose the AlcPinA strain grows at a slower rate than that of the Pal5 control strain as determined by the increase in average number of nuclei per germling as a function of time (Figure 2C). In contrast, in the presence of glycerol or glycerol plus threonine both strains grew at the same rate (Figure 2C and data not shown). Using the data presented in Figure 2C, the exponential rate of
nuclear division the AlcPinA strain grown in dextrose is calculated to be approximately 95 min. versus 75 min. for the control. In glycerol, both strains have a doubling time of 125 min. Importantly, Figure 2C clearly demonstrates that the growth delay due to PINA repression is not due to a lag in germination as the slope of the growth curve is decreased when plotted in a log format. Thus, although carbon source-mediated repression of PINA expression is not sufficient to mimic the effects of pinA disruption, the reduction of endogenous PINA levels to approximately 2-percent of wild-type significantly decreases the rate of nuclear division.

**PINA is required for normal G1 progression**

Since Pin1 has been suggested to play an important role in multiple phases of the cell cycle, we evaluated whether reduced PINA expression caused a specific delay in cell cycle progression in *A. nidulans*. As Pin1 was initially identified as a negative regulator of NIMA function we first analyzed whether reduced PINA levels altered S-phase and G2 progression. First we generated new strains of *A. nidulans* by crossing either the AlcPinA or the control strain with a strain containing a temperature sensitive mutation in the G1 cyclin, *nimG* (31). When these new strains were arrested in G1 at the restrictive temperature and released into a permissive temperature the timing of mitotic entry as indicated by chromosome mitotic index (Figure 3A) or histone H3 ser10 phosphorylation (Figure 3B) were unaffected by reduced PINA levels, suggesting that the reduced rate of nuclear division observed in the AlcPinA strain is not due to a lengthening of S or G2 phase.

As a reduction in PINA levels have been reported to induce a mitotic arrest in both mammalian and fungal systems, we next asked whether the *pinA*-induced reduction
in proliferation was due to a lengthening of mitosis. To address this question we crossed
the AlcPINa strain with a strain harboring a temperature sensitive mutation in cyclinB
\((nimE)\) and followed mitotic progression when released from the G2 arrest. As seen in
Figure 3C, when AlcPINa and nutritionally complemented controls are released from the
\(nimE\) arrest point under conditions that repress PINA expression, the cells progress
through mitosis at the same rate as determined by following chromosome mitotic index
or the appearance of multinucleate germlings. Thus, since \(pinA\) repression does not
appear to cause a reduction in the rate of progression through S, G2 or mitosis it is likely
that the observed increase in the nuclear division cycle due to \(pinA\) repression is due to a
lengthening of G1.

To determine whether the reduced PINA levels did alter G1 progression we
examined the changes in NIMX\(^{cdc2}\) kinase activity as a function of time after germination.
AlcPinA and Pal5 were germinated in medium containing dextrose and germinating
conidia were collected for analysis every half hr from 2 through 4.5 hr post-germination.
As demonstrated in Figure 3D the activation of NIMX\(^{cdc2}\) histone H1 kinase activity is
delayed by approximately 30 min. under conditions which repress PINA expression. The
approximate 30 min. delay in NIMX\(^{cdc2}\) activation correlates well with the approximate
20 min. lengthening of the cell cycle under conditions which repress PINA expression.
Thus, reduced PINA levels appear to cause a lengthening of the cell cycle by increasing
the duration of G1 prior to NIMX\(^{cdc2}\) activation.

PINA functions in the recovery from S-phase arrest

Since PINA was isolated as a negative regulator of NIMA activity we surmised it
would likely play some role in G2/M progression in \(A. nidulans\). To test this idea we
arrested the AlcPinA and control strains in S-phase with hydroxyurea and then examined entry into mitosis following release from the drug by chromosome mitotic index or the temporal accumulation of histone H3 ser10 phosphorylation. In contrast to the results observed upon release from the nimG arrest presented earlier, the AlcPinA strain accumulated condensed chromatin and ser10 phosphorylated histone H3 approximately 10 to 20 min. earlier than the Pal5 strain following release from the hydroxyurea arrest (Figure 4A and B).

Since reduction of PINA levels accelerate mitotic entry following a hydroxyurea release we evaluated if this apparent alteration in the replication checkpoint also resulted in an increased sensitivity to hydroxyurea. If PINA performs a critical role in establishing or maintaining the replication checkpoint in *A. nidulans* it would likely be reflected by an increased sensitivity to hydroxyurea. As seen in Figure 4C, AlcPinA and Pal5 appear to have the same sensitivity to increasing concentrations of hydroxyurea, indicating that PINA does not cause a defect in initiating the replication arrest induced by hydroxyurea. Reduced PINA levels also had no effect on sensitivity to other agents that result in various checkpoint arrests such as UV (Figure 4D) or benomyl (data not shown). Thus, it appears that reduced PINA levels do not inhibit initiation of the replication checkpoint arrest but perhaps are important for recovery from the arrest.

**PINA physically interacts with NIMA**

As PINA was originally identified as a NIMA interacting protein via the two-hybrid assay (17) we asked whether the two proteins interact *in vivo*. To address this question we performed coimmunoprecipitation assays utilizing a strain of *A. nidulans* expressing one extra copy of HA-tagged NIMA and harboring a temperature sensitive
mutation in nim\textsuperscript{Tcd25} (CDS46) (30). As demonstrated in Figure 5A, when HA-NIMA was immunoprecipitated from extracts derived from asynchronous, benomyl or nim\textsuperscript{T} arrested extracts, PINA can be coimmunoprecipitated. Interestingly, the amount of PINA coprecipitated is greatest in the benomyl arrested extract indicating that, similar to Pin1, PINA associates most avidly with mitotically phosphorylated NIMA. Likewise, when PINA is immunoprecipitated from benomyl-arrested fungus, HA-NIMA is also present in the precipitate (Figure 5A).

Since PINA appears to associate most avidly with mitotically phosphorylated NIMA we next examined what domains of both PINA and NIMA were required for this interaction. To address this question we utilized a traditional yeast two-hybrid assay and scored positive interactions by promotion of GAL4-dependent transcription of HIS3, which compliments the \textit{his3-200} deletion and allows yeast proliferation on medium in the absence of histidine. As observed in the original interaction screen, full-length PINA fused to the GAL4 transcriptional activation domain interacts with full-length NIMA fused to the GAL4 DNA binding domain since yeast expressing these two proteins grow equally well in the presence or absence of histidine (Figure 5B). Additionally, mutation of the PINA WW-domain (W33A) or expression of the prolyl-isomerase domain (PI domain) alone does not alter the ability of the yeast to grow on His- medium. However, either mutation of the prolyl-isomerase active site (C126A) or removal of the prolyl-isomerase domain completely (WW-domain) abolishes the ability of the yeast to grow in the absence of histidine without altering its proliferative properties in medium supplemented with histidine. All the proteins were expressed to similar levels within the yeast strains (data not shown). Thus, in contrast to the characterized interactions of Pin1
and its target proteins, the interaction of PINA with NIMA is dependent upon a functional prolyl-isomerase domain and the WW-domain appears to be dispensable for the interaction.

To determine the domain(s) of NIMA required for its interaction with PINA we performed two-hybrid analysis of wild-type PINA with various NIMA fragments. Consistent with results using Pin1, the catalytic domain of NIMA (NIMA 1-292) fused to the Gal4 DNA binding domain does not support yeast growth in the absence of histidine while the C-terminus (NIMA 293-699) does interact with PINA in the two-hybrid assay. Since the Pin1 interaction domain of NIMA was originally identified to lie within amino acids 280-396 (14), we tested whether PINA interacts with a similar fragment, NIMA 293-396. Figure 5C demonstrates that NIMA 293-396 does not interact with PINA in the two-hybrid system while the C-terminal fragment containing amino acids 397-699 demonstrates a positive interaction in this system. All NIMA fragments were expressed to similar levels in the yeast (data not shown). Thus, the PINA binding domain of NIMA appears to be confined to the C-terminal 302 amino acids of the protein.

**PINA positively regulates NIMA function in Vivo**

Since Pin1 was originally identified as a NIMA interacting protein and demonstrated to negatively regulate NIMA function in cultured cells (14), we examined whether PINA regulated NIMA function in *A. nidulans*. Because PINA repression or overexpression does not appear to share any phenotypic consequences with NIMA repression or overexpression in this organism, we tested for a genetic interaction between PINA and NIMA. Such an interaction was examined by crossing the AlcPinA strain with a strain containing the nimA5 temperature sensitive mutation and testing whether the
repression or overexpression of PINA altered the temperature sensitive growth of this new strain. As shown in Figure 6, in the absence of the nimA5 mutation (Wt.), the AlcPinA and Pal5 strains grow on the various carbon sources as described earlier, and display little to no temperature-dependent growth variation at 31°, 37° or 41°C. However, in the nimA5 background there are two significant growth differences. First, when germinated in the presence of dextrose to repress PINA expression, the nimA5/AlcPinA strain did not grow even at the nimA5 permissive temperature of 31°C. In contrast, at 31°C in dextrose the nimA5/Pal5 strain grows similar to the wild-type/Pal5 control strain. Second, when the strains are germinated on glycerol plus threonine to induce PINA overexpression, the AlcPinA/nimA5 strain shows enhanced growth compared to the Pal5/nimA5 control strain when germinated at the partially permissive temperature of 37°C. Thus, PINA positively regulates NIMA function genetically. Specifically, reduced PINA expression exacerbated the nimA5 temperature sensitive mutation while PINA overexpression partially relieves the temperature sensitivity of growth. Moreover, this genetic interaction is selective for NIMA since no genetic interactions were seen with temperature sensitive mutations of other genes involved in growth control including NIMT<sup>cdc25</sup>, NIMQ<sup>mcm2</sup> or NIME<sup>cyclinB</sup> (data not shown).

**PINA does not regulate NIMA kinase activity**

Since PINA not only interacts with NIMA but also positively influences its function, we evaluated whether PINA altered NIMA’s kinase activity *in vitro* or *in vivo*. To assess the effect of PINA on NIMA kinase activity *in vitro* we tested whether the addition of purified PINA altered the kinase activity of bacterially expressed and purified full length NIMA. As seen in Figure 7A, in comparison to BSA, the addition of PINA...
has no effect on NIMA kinase activity, using a peptide substrate, either before or after an activating preincubation in the presence of ATP. Additionally, the presence of PINA did not alter the Km for either peptide or ATP substrates (data not shown).

Given that Pin1 has been demonstrated to promote PP2A function (38), and PP2A has also been shown to dephosphorylate and inactivate NIMA in vitro (39), we next examined whether the addition of PINA would facilitate the ability of PP2A to dephosphorylate and inactivate recombinant NIMA. To test whether PINA promotes PP2A dephosphorylation of NIMA we followed the dephosphorylation of active bacterially expressed NIMA in the presence of added PINA or BSA through the use of the MPM2 monoclonal antibody which recognizes antigens similar to those that bind Pin1. As demonstrated in Figure 7B, the addition of PINA or BSA alone has no effect on the phosphorylation state of recombinant NIMA over a 60 min. incubation. However, the addition of purified PP2A results in a significant time-dependent reduction in MPM2 immunoreactivity independent of the presence of PINA. Similarly, in comparison to BSA, the presence of PINA does not alter the ability of PP2A to promote inactivation of NIMA kinase activity in vitro (Figure 7C). Thus, PINA does not appear to alter either NIMA activity or PP2A mediated dephosphorylation of NIMA in vitro.

Finally, although we could detect no deficits in the G2/M transition under conditions which repress PINA expression, we questioned whether PINA repression altered NIMA protein levels or kinase activity. In order to more easily detect and assay NIMA from A. nidulans extracts we crossed the AlcPinA strain with the CDS46 strain, which possesses one extra copy of HA tagged NIMA and used this new strain in addition to the parental CDS46 strain for the subsequent experiments. First, we assayed NIMA
protein levels and kinase activity in extracts derived from mitotically arrested AlcPINA/CDS46 and CDS46 under conditions which repress, de-repress or induce PINA expression. Figure 7D demonstrates that in mitotically-arrested germlings HA-NIMA levels are constant regardless of carbon source or PINA levels. Additionally, there is very little carbon source or PINA-dependent variation in HA-NIMA kinase activity when HA-immunoprecipitates are assayed in a peptide kinase assay (Figure 7E). Therefore, under the conditions of the assays, PINA repression or overexpression has little or no effect on either NIMA protein level or kinase activity in vivo.

DISCUSSION

Although Pin1 has been implicated in numerous signaling pathways critical for cell proliferation in multiple eukaryotic systems, the phenotypic responses to Pin1 deletion or inhibition vary greatly among these systems. Pin1 is essential for growth in Saccharomyces cerevisiae and Candida albicans but its disruption in Schizosaccharomyces pombe, mouse and Drosophila has less severe phenotypic consequences (4-12). Here we report characterization of the in vivo roles of the A. nidulans Pin1 homologue, PINA.

Similar to S. cerevisiae and C. albicans, PINA is essential for A. nidulans cell proliferation (4,5). Although we were unable to characterize the terminal phenotype of the PINA null fungus, a 50-fold reduction of PINA protein increases the doubling time of A. nidulans by approximately 25%. Based on the consistent increase in cell cycle length between the first nuclear division and subsequent divisions and the lack of evidence that other phases of the cell cycle are altered in length, we believe that the reduced growth rate under conditions which repress pinA expression is due to an increase in the length of
G1. However, we cannot exclude the possibility that the observed lengthening of the cell cycle when PINA is repressed could be due to a subtle lengthening of multiple cell cycle phases which are unable to be detected by the assays used. Nevertheless, our findings contrast with the observations that temperature sensitive mutations in the *S. cerevisiae* and *C. albicans* Pin1 homologue, *ess1*, lead to a mitotic arrest (5,13). However, data suggest that the essential mitotic role of *ess1p* in *S. cerevisiae* is not direct (i.e. acting directly upon a regulator of mitosis) but instead is due to alterations in transcriptional regulation (13). Regardless, although we interpret our data to indicate that PINA function is critical for the proper timing of G1, they do not preclude the possibility that a rate-limiting amount of PINA (less than 2% of normal cells) is essential for progression through other phases of the cell cycle such as mitosis.

A G1 role for PINA in *A. nidulans* is consistent with reported G1 deficits observed in mammalian cell systems. Although expression of anti-sense Pin1 RNA in HeLa cells has been reported to induce a cell cycle arrest with a high percentage of cells containing condensed chromatin (14), the characterization of Pin1 null mice and fibroblasts has produced evidence for a critical role of Pin1 for G1 progression. Pin1 null primordial germ cells and MEFs grow at a slower rate compared to controls and MEFs are impaired in cell cycle re-entry following serum deprivation (9,11,12,18). At the biochemical level, Pin1 has been suggested to positively regulate numerous proteins important for or implicated in G1 progression including cyclinD1, c-jun, and β-catenin (10,19,20). Although the mechanism by which Pin1 modulates the function of these proteins varies from changes in activity to stability to localization, a consistent theme in the G1 roles proposed for Pin1 is its involvement in transcriptional regulation (reviewed...
in 40) Thus, an obvious question is whether the G1 phenotype we have observed in *Aspergillus* is reflective of a role Pin1 in the transcriptional regulation of genes important for G1 control. If this is the case, the phenotype observed in *A. nidulans* could be more similar to the role *ess1* performs in *S. cerevisiae* than might have initially appeared to be so. In both systems Pin1 may be altering the transcriptional regulation of genes critical for cell cycle progression but in budding yeast this is manifest by a mitotic arrest while *A. nidulans* displays a G1 deficit. Unfortunately, little is known about either transcriptional regulation or the genes involved in G1 progression in *A. nidulans*, thus elucidation of the molecular target(s) of Pin1 responsible for or contributing to this phenotype remains to be addressed by future work.

Similar to results obtained in *S. pombe* but in contrast to Pin1 null fibroblasts, reduced PINA protein levels do not appear to markedly alter *A. nidulans*’ response to DNA damaging agents (UV-irradiation or MMS), or the replication inhibitor hydroxyurea (6,41-43). However, following release from a hydroxyurea induced replication arrest, the fungal cells enter mitosis more rapidly under conditions that repress PINA expression, a result reminiscent of our previous data that depletion of Pin1 from interphase Xenopus egg extracts accelerates the entry into mitosis upon addition of cyclinB (44). If, as observed in Xenopus egg extracts, PINA performs a critical role in establishing or maintaining the replication checkpoint in *A. nidulans*, it would likely be reflected by an increased sensitivity to hydroxyurea. However, because PINA does not alter the sensitivity to hydroxyurea we interpret these results to suggest that PINA plays a role in the recovery from a checkpoint-induced cell cycle arrest rather than in the establishment or maintenance of the replication checkpoint. In *A. nidulans* the replication
checkpoint has two described components (45-47). The first, initiated when DNA replication is slowed, results in the inhibitory tyrosine phosphorylation of NIMX\text{cdc2} via ANKAw\text{eel} which is relieved by NIMT\text{cdc25} (46,47). Although both \text{eel} and \text{cdc25} have been demonstrated to interact with Pin1 (16,17), we believe this component of the checkpoint pathway to be intact as the fungus shows no increased sensitivity to hydroxyurea. The second component of the checkpoint is enabled when replication is completely inhibited and is dependent upon the activity of BIME\text{APC1} (45,46). The current hypothesis is that BIME\text{APC1} prevents the lethal accumulation and activation of NIMA during the replication arrest. Thus, even though both components of the checkpoint pathway are functional, either (or both) arm(s) of the pathway could be subject to PINA regulation yielding the observed acceleration into mitosis following release from a hydroxyurea arrest.

Consistent with previous reports of the interaction of Pin1 with NIMA, by coimmunoprecipitation PINA appears to associate most avidly with the mitotic form of NIMA. Upon mitotic entry NIMA not only becomes hyperphosphorylated on numerous S/TP motifs presumably via NIMX\text{cdc2}, but also changes subcellular localization from perinuclear to nuclear (30,48). In contrast to the defined interaction domain with Pin1 (14), we have mapped the PINA interaction domain of NIMA to the C-terminal 302 amino acids which contain the majority of the potential mitotically phosphorylated S/TP sites. Given that Pin1 has been demonstrated to interact with mitotic phosphoproteins on sites which can be generated by cdc2 kinase activity and that the primary cellular localization of PINA is nuclear, the increased association of PINA and NIMA in benomyl-arrested extracts is likely due to both an increased phosphorylation state of the
protein as well as its transport into the nucleus where PINA is concentrated. However, although our data suggest that the NIMA/PINA interaction is phosphorylation dependent, this question has yet to be addressed specifically.

Although the association of PINA with NIMA via coimmunoprecipitation came as no surprise to us and confirmed the original identification of the interaction via the 2-hybrid system (17), the results from mapping of the domains responsible for the interaction were quite unexpected. Not only did the PINA binding site map to the C-terminal 302 amino acids of NIMA, but the primary domain in PINA responsible for its interaction with NIMA is the prolyl-isomerase domain. Based on the precedent that the domain responsible for the interaction of Pin1 with its target proteins is the WW-domain, the findings that the WW-domain of PINA binds many phosphorylated peptides known to bind Pin1 and that PINA interacts primarily with the mitotic form of NIMA, we would have predicted that the WW-domain of PINA would also be responsible for its interaction with NIMA. These results raise the question as to whether this type of interaction is specific to PINA or could also reflect the presence of a different class of Pin1-interacting proteins. The isomerase domain-dependent interaction of PINA with NIMA is reminiscent of numerous characterized immunophilin complexes including (but not limited to) FKBP12’s interactions with ryanodine, IP3 and TGFβ receptors, the cyclophilinA:HIV capsid p24 and Gag protein complexes and the cyclophilinB:CAML protein complex (49-53). In instances such as these it has been proposed that the rotomase recognizes and binds with high affinity to a domain of the target protein which structurally resembles conformation of a suitable isomerization substrate with some subtle differences (reviewed in 54).
That PINA interacts with NIMA both physically and genetically is clear from our study although the molecular consequences of this interaction remain enigmatic. Pin1 has been demonstrated to affect protein function by a number of mechanisms including alteration of protein stability, localization, transcription and enzymatic activity (reviewed in 1,2), all of which are known modes of NIMA regulation \textit{in vivo}. Although measured statically and under conditions designed to produce maximal activity using a non-physiological peptide substrate, our data suggest that PINA does not significantly alter NIMA protein levels or kinase activity. Thus, it seems possible that PINA plays a non-essential role in targeting the subcellular localization of NIMA. For example, PINA could act as an adaptor molecule by targeting NIMA to mitotic phosphoproteins. In this case, the prolyl-isomerase domain could play a non-catalytic role acting as a phosphorylation state specific binding domain, a role normally attributed to the WW-domain, and the WW-domain could act in trans to effect formation of a ternary, phosphorylation-dependent, protein complex. Although multi-domain adaptor molecules targeting phosphoepitopes are quite common, this function has yet to be proposed for Pin1. Structurally, Pin1 has been proposed to be suitable for such a signal dependent adaptor molecule as the WW-domain and prolyl-isomerase domain are connected by a highly flexible linker that can adapt different conformations depending upon the peptide bound (55). Thus, by this model PINA could influence NIMA function by facilitating the proper subcellular localization of the active kinase to sites that require phosphorylation by NIMA. Clearly, this mechanism has yet to be tested and there are other potential mechanisms by which PINA could positively regulate NIMA function \textit{in vivo}. 
Pin1-mediated, phosphorylation-directed proline isomerization is now appreciated to be a conserved mechanism for modulation of cellular signaling events in organisms from *S. cerevisiae* to mammals. The demonstration that Pin1/Ess1p/PINA is essential for proliferation in *S. cerevisiae*, *C. albicans* and *A. nidulans* emphasizes the emerging importance of phosphorylation-dependent conformational changes mediated via proline isomerization. Although the deletion of Pin1 is not universally lethal (mice, *D. melanogaster* and *S. pombe*) there is evidence suggesting that other isomerases can substitute for Pin1 in its absence. Drugs targeting the catalytic activity of both Pin1 and a parvulin family member, par14, have been demonstrated to block proliferation of numerous mammalian cell lines (56). Also, in *S. pombe* the disruption of Pin1 sensitizes the yeast to the cyclophilin inhibitor, cyclosporin A (6,57). Given the difficulty presented by the apparent genetic redundancy in multicellular organisms and the multitude of potential Pin1 targets, the characterization of its essential roles in a genetically tractable system such as *A. nidulans* should yield mechanistic insight into the critical conserved cellular roles of Pin1-mediated proline isomerization in more complex metazoans.

**ACKNOWLEDGMENTS**

We thank Christina R. Kahl for valuable discussions and critical reading of the manuscript. We also thank Dr. Stephen A. Osmani and Dr. Steven W. James for generously providing many *A. nidulans* strains used in this study. This work was supported by an NIH training grant 2T32DK07568-13 (JDJ) and research grant CA82845 (ARM).
REFERENCES

1. Lu, K. P., Liou, Y. C., and Zhou, X. Z. (2002) *Trends Cell Biol* **12**(4), 164-72.

2. Joseph, J. D., Yeh, E. S., Swenson, K. I., and Means, A. R. (2003) in *Progress in Cell Cycle Research* (Meijer, L., Jezequel, A., and Roberge, M., eds) Vol. Vol. 5, pp. 477-487, Life in Progress Editions, Roscoff, France.

3. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J. U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) *Science* **278**(5345), 1957-60.

4. Hanes, S. D., Shank, P. R., and Bostian, K. A. (1989) *Yeast* **5**(1), 55-72.

5. Devasahayam, G., Chaturvedi, V., and Hanes, S. D. (2002) *Genetics* **160**(1), 37-48.

6. Huang, H. K., Forsburg, S. L., John, U. P., O'Connell, M. J., and Hunter, T. (2001) *J Cell Sci* **114**(Pt 20), 3779-88.

7. Maleszka, R., Hanes, S. D., Hackett, R. L., de Couet, H. G., and Miklos, G. L. (1996) *Proc Natl Acad Sci U S A* **93**(1), 447-51.

8. Hsu, T., McRackan, D., Vincent, T. S., and Gert de Couet, H. (2001) *Nat Cell Biol* **3**(6), 538-43.

9. Fujimori, F., Takahashi, K., Uchida, C., and Uchida, T. (1999) *Biochem Biophys Res Commun* **265**(3), 658-63.

10. Liou, Y. C., Ryo, A., Huang, H. K., Lu, P. J., Bronson, R., Fujimori, F., Uchida, T., Hunter, T., and Lu, K. P. (2002) *Proc Natl Acad Sci U S A* **99**(3), 1335-40.

11. Atchison, F. W., Capel, B., and Means, A. R. (2003) *Development* **130**(15), 3579-86.
12. Atchison, F. W., and Means, A. R. (2003) Biol Reprod 69(6), 1989-97
13. Wu, X., Wilcox, C. B., Devasahayam, G., Hackett, R. L., Arevalo-Rodriguez, M., Cardenas, M. E., Heitman, J., and Hanes, S. D. (2000) Embo J 19(14), 3727-38.
14. Lu, K. P., Hanes, S. D., and Hunter, T. (1996) Nature 380(6574), 544-7.
15. Rippmann, J. F., Hobbie, S., Daiber, C., Guilliard, B., Bauer, M., Birk, J., Nar, H., Garin-Chesa, P., Rettig, W. J., and Schnapp, A. (2000) Cell Growth Differ 11(7), 409-16.
16. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) Genes Dev 12(5), 706-20.
17. Crenshaw, D. G., Yang, J., Means, A. R., and Kornbluth, S. (1998) Embo J 17(5), 1315-27.
18. You, H., Zheng, H., Murray, S. A., Yu, Q., Uchida, T., Fan, D., and Xiao, Z. X. (2002) J Cell Biochem 84(2), 211-6
19. Wulf, G. M., Ryo, A., Wulf, G. G., Lee, S. W., Niu, T., Petkova, V., and Lu, K. P. (2001) Embo J 20(13), 3459-72.
20. Ryo, A., Nakamura, M., Wulf, G., Liou, Y. C., and Lu, K. P. (2001) Nat Cell Biol 3(9), 793-801.
21. Miyashita, H., Mori, S., Motegi, K., Fukumoto, M., and Uchida, T. (2003) Oncol Rep 10(2), 455-61.
22. Osmani, S. A., Pu, R. T., and Morris, N. R. (1988) Cell 53(2), 237-44.
23. Lu, K. P., and Means, A. R. (1993) Meth. Mol. Gen. 2, 255-275
24. Pontecorvo, G. (1953) in Advances in Genetics (Demerec, M., ed), pp. 141-238, Academic Press, New York
25. Guthrie, C., and Fink, G. R. (2002) *Guide to Yeast Genetics and Molecular and Cell Biology*. Methods in Enzymology (Abelson, J. N., and Simon, M. I., Eds.), 351, Academic Press, New York
26. Waring, R. B., and May, G. S. (1989) *Gene* 79, 119-130
27. Rasmussen, C. D., Means, R. L., Lu, K. P., May, G. S., and Means, A. R. (1990) *J. Biol. Chem.* 265(23), 13767-75
28. Harris, S. D., Morrell, J. L., and Hamer, J. E. (1994) *Genetics* 136, 517-532
29. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. De Souza, C. P., Osmani, A. H., Wu, L. P., Spotts, J. L., and Osmani, S. A. (2000) *Cell* 102(3), 293-302.
31. Dayton, J. S., Sumi, M., Nanthakumar, N. N., and Means, A. R. (1997) *Journal of Biological Chemistry* 272(6), 3223-30
32. Sarkar, G., and Sommer, S. S. (1990) *Biotechniques* 8(4), 404-407
33. Joseph, J. D., and Means, A. R. (2000) *J. Biol. Chem.* 275, 38230-38238
34. Dayton, J. S., and Means, A. R. (1996) *Mol. Biol. Cell* 7(10)), 1511-9
35. Rasmussen, C., Garen, C., Brining, S., Kincaid, R. L., Means, R. L., and Means, A. R. (1994) *EMBO* 13(16)), 3917-24
36. Lu, K. P., Rasmussen, C. D., May, G. S., and Means, A. R. (1992) *Mol. Endo.* 6(3), 365-74
37. McGoldrick, C. S., Gruver, C., and May, G. S. (1995) *J. Cell Biol.* 128(4), 577-587
38. Zhou, X. Z., Kops, O., Werner, A., Lu, P. J., Shen, M., Stoller, G., Kullertz, G., Stark, M., Fischer, G., and Lu, K. P. (2000) Mol Cell 6(4), 873-83.
39. Lu, K. P., Osmani, S. A., and Means, A. R. (1993) J Biol Chem 268(12), 8769-76
40. Shaw, P. E. (2002) EMBO Rep 3(6), 521-6
41. Zheng, H., You, H., Zhou, X. Z., Murray, S. A., Uchida, T., Wulf, G., Gu, L., Tang, X., Lu, K. P., and Xiao, Z. X. (2002) Nature 419(6909), 849-53.
42. Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and Del Sal, G. (2002) Nature 419(6909), 853-7.
43. Wulf, G. M., Liou, Y. C., Ryo, A., Lee, S. W., and Lu, K. P. (2002) J Biol Chem 277(50), 47976-9.
44. Winkler, K. E., Swenson, K. I., Kornbluth, S., and Means, A. R. (2000) Science 287(5458), 1644-7.
45. James, S. W., Mirabito, P. M., Scacheri, P. C., and Morris, N. R. (1995) J Cell Sci 108(Pt 11), 3485-99.
46. Ye, X. S., Fincher, R. R., Tang, A., O'Donnell, K., and Osmani, S. A. (1996) Embo J 15(14), 3599-610.
47. De Souza, C. P., Ye, X. S., and Osmani, S. A. (1999) Mol Biol Cell 10(11), 3661-74.
48. Ye, X. S., Xu, G., Pu, R. T., Fincher, R. R., McGuire, S. L., Osmani, A. H., and Osmani, S. A. (1995) Embo J 14(5), 986-94.
49. Jayaraman, T., Brillantes, A. M., Timerman, A. P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and Marks, A. R. (1992) J Biol Chem 267(14), 9474-7
50. Cameron, A. M., Steiner, J. P., Sabatini, D. M., Kaplin, A. I., Walensky, L. D., and Snyder, S. H. (1995) Proc Natl Acad Sci U S A 92(5), 1784-8

51. Wang, T., Donahoe, P. K., and Zervos, A. S. (1994) Science 265(5172), 674-6

52. Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993) Cell 73(6), 1067-78

53. Bram, R. J., and Crabtree, G. R. (1994) Nature 371(6495), 355-8

54. Ivery, M. T. (2000) Med Res Rev 20(6), 452-84

55. Jacobs, D. M., Saxena, K., Vogtherr, M., Bernado, P., Pons, M., and Fiebig, K. (2003) J Biol Chem 278(128), 26174-26182

56. Uchida, T., Takamiya, M., Takahashi, M., Miyashita, H., Ikeda, H., Terada, T., Matsuo, Y., Shirouzu, M., Yokoyama, S., Fujimori, F., and Hunter, T. (2003) Chem Biol 10(1), 15-24.

57. Fujimori, F., Gunji, W., Kikuchi, J., Mogi, T., Ohashi, Y., Makino, T., Oyama, A., Okuhara, K., Uchida, T., and Murakami, Y. (2001) Biochem Biophys Res Commun 289(1), 181-90.

FIGURE LEGENDS

Figure 1: pinA is essential for A. nidulans proliferation. A. Shown is a schematic representation of the homologous integration of the pPINAdis disruption plasmid into the endogenous pinA locus. The approximate location of the HindIII restriction sites used for identification of the homologously integrated transformants are noted. B. Diagram of the heterokaryon transformation strategy. Homologous integration of pPINAdis into the
pinA locus generates a heterokaryon containing nuclei with the genotypes pry4+pinA− and pry4pinA+. Following asexual reproduction the uninucleate spores maintain the genotypes pry4+pinA− and pry4−pinA+. If PINA is essential neither genotype will germinate in rich medium (YG) and only pry4pinA+ will germinate in rich medium containing uracil and uridine. C. Southern analysis of ΔpinA/pinA heterokaryons. Southern analysis of HindIII digested total genomic DNA derived from the parental (GR5) and heterokaryon demonstrates the presence of the predicted 0.9 Kbp fragment representing the endogenous pinA locus and the additional 3.0 Kbp bands in the homologously integrated transformants. D. Growth of spores derived from ΔpinA/pinA heterokaryon. Spores derived from a single heterokaryon strain were inoculated into either YG or YG plus uridine and uracil. The data shown are representative of results obtained from 6 heterokaryon strains.

Figure 2: Repressed PINA expression results in decreased rate of A. nidulans nuclear division. A. A representation of the homologous integration of pAlcPinA into the endogenous pinA locus. B. Western analysis of PINA protein levels in the AlcPinA strain in comparison to the nutritionally complemented control (Pal5). Strains were germinated in minimal medium containing either dextrose (repressing), glycerol (non-repressing) or glycerol plus threonine (inducing) protein was extracted from exponentially growing cells. The AlcPinA protein levels are expressed as % expression relative to the control when cells are grown in the same carbon source. C. Nuclear number of AlcPinA (circles) and Pal5 (squares) germinated in minimal medium plus dextrose (closed) or glycerol (open). The inset shows the same data presented as a log
scale plot. Each time point represents the average of at least 150 germlings, the error bars represent the S.E.M. The data presented is representative of at least 3 independent experiments.

Figure 3: PINA repression delays the S-phase activation of NIMX^{cdc2}. A. PINA repression does not alter the length of S-phase and G2. AlcPinA + nimG10 and Pal5 + nimG10 were inoculated into minimal medium plus dextrose. Exponentially growing cells were then arrested at the G1/S boundary by incubation at 42°C for 2 hr. Following release, samples were collected and the chromosome mitotic index determined. B. PINA repression does not alter the time course of histone H3 ser10 phosphorylation following release from nimG arrest. As in Fig. 3A, germlings were arrested at the restrictive temperature and released at 30°C. Samples were collected and analyzed for histone H3 ser10 phosphorylation. C. PINA repression does not alter mitotic progression following nimE arrest. AlcPinA + nimE6 (squares) and Pal5 + nimE6 (circles) were inoculated into minimal medium plus dextrose and arrested in late G2 by incubation at 42°C. Following release CMI (open figures) and % germlings with >1 nuclei (closed figures) were determined. D. PINA repression delays NIMX^{cdc2} activation upon germination. AlcPinA (squares) and Pal5 (circles) were germinated in minimal medium dextrose to repress PINA protein expression. Following 2 hr of growth samples were taken every 30 min. until 4.5 hr post-germination. Extracts from the samples were assayed for NIMX^{cdc2} histone H1 kinase activity following p13-agarose precipitation as described in the “Experimental Methods”. The data are representative of three independent experiments.
Between experiments the absolute timing of mitotic entry varied by as much as 20 min. but the differences between the AlcPINA and Pal5 strains were consistent.

Figure 4: The absence of PINA accelerates recovery from a hydroxyurea-induced arrest without altering drug sensitivity. A. PINA repression accelerates entry into mitosis following a hydroxyurea-induced arrest. Exponentially growing AlcPinA and Pal5 germlings were arrested in hydroxyurea for 2 hr and released into fresh medium. Following release samples were taken every 10 min. for analysis of CMI as described in “Experimental Procedures”. B. PINA repression accelerates accumulation of histone H3 Ser10 phosphorylation following release from hydroxyurea-induced arrest. Exponentially growing AlcPinA and Pal5 germlings were arrested in hydroxyurea for 2 hr and released into fresh medium. Following release samples were taken every 10 min. for analysis of histone H3 phosphorylation. C. Reduced PINA expression does not alter the sensitivity of A. nidulans to hydroxyurea. AlcPinA and Pal5 were germinated on minimal medium plus dextrose or glycerol in the presence of hydroxyurea and allowed to germinate for 2 days at 37°C. D. PINA repression does not alter the sensitivity of A. nidulans to UV irradiation. Pal5 (circles) and AlcPinA (squares) germlings were irradiated 4 hr post germination, followed by plating on minimal medium plus glycerol or dextrose and the % survival after 3 days at 37°C was determined. All results are representative of those obtained in at least 3 independent experiments.

Figure 5: NIMA and PINA physically interact. A. NIMA and PINA interact via coimmunoprecipitation. HA-NIMA was immunoprecipitated from extracts of Pal5 or
CDS46 either asynchronous, mitotically arrested (benomyl) or G2 arrested (43°C) followed by western analysis for HA-NIMA and PINA. PINA was immunoprecipitated from benomyl-arrested CDS46 followed by western analysis of both PINA and HA-NIMA. The results are representative of multiple experiments. B. A functional prolyl-isomerase domain is required for PINA to interact with NIMA. *S. cerevisiae* transformants of pAD-PINA and various mutants and truncations and pBD-NIMA were serially diluted and spotted on CSM lacking either trp and leu or trp, leu, and his. C. PINA interacts with the C-terminus of NIMA. *S. cerevisiae* transformants of pAD-PINA and pBD-NIMA and various NIMA truncations were diluted and spotted on to CSM and described above. These results are representative of at least 2 experiments performed with at least 4 strains of each genotype.

Figure 6: PINA displays a positive genetic interaction with NIMA. Pal5, AlcPinA, Pal5 + nimA5 and AlcPinA + nimA5 were inoculated onto minimal medium containing dextrose (repressing), glycerol (non-repressing) or glycerol plus threonine (inducing) and incubated at the indicated temperatures for 2 or 3 days. These data are representative of 3 experiments with 3 strains of each genotype.

Figure 7: PINA does not alter NIMA activity, dephosphorylation of protein levels. A. PINA does not alter the kinase activity of recombinant NIMA. Preincubated or non-preincubated NIMA was incubated with either PINA or BSA followed by *in vitro* kinase assay. B. PINA does not alter PP2A-mediated NIMA dephosphorylation. Recombinant NIMA was incubated in the presence of NIMA or PINA followed by the addition of
PP2A. Dephosphorylation of potential PINA target sites was monitored via MPM2 immunoblot over a period of 1 hr. C. PINA does not potentiate PP2A-mediated NIMA inactivation. NIMA activity was followed over a time course of PP2A treatment in the presence or absence of PINA. D. PINA repression of induction does not alter HA-NIMA protein levels. CDS46 and AlcPINA X CDS46 were germinated in minimal medium plus either dextrose (D), glycerol (G) or glycerol plus threonine (G+T) and mitotically arrested with the addition of benomyl. HA-NIMA and PINA expression levels were observed by western analysis. E. PINA repression of induction does not alter the kinase activity of HA-NIMA. HA-NIMA activity was analyzed following immunoprecipitation from extracts derived as in Fig 7D. All data presented are representative of at least 3 independent experiments.
Figure 1

A.

Wild type pinA locus

Disrupted pinA locus

0.9Kbp. HindIII Fragment

2 3.0Kbp. HindIII Fragments

B.

pPINAdis

fuse or multinucleate

pyr4<sup>+</sup> pinA<sup>+</sup>

pyr4<sup>-</sup> pinA<sup>-</sup>

C.

Kbp.

3.0

0.9

ApinA/pinA

ApinA/pinA

GR5

D.

ΔpinA/pinA

Percent germinated

54%

<1%
Figure 2

A. [Diagram showing the pAlcPINA vector with EcoR1 sites and genetic elements.

Wild type pinA locus

pAlcPINA

EcoR1

pinA locus in the AlcPinA strain

EcoR1

pinA 3' deletion

pyr4

Alc:pinA Hybrid Gene

EcoR1

3.4 Kbp. EcoR1 Fragment

7.6 and 1.8 Kbp. EcoR1 Fragments

B. [Table showing AlcPINA and Pal5 expression in MMD, MMG, and MMG+T conditions.

|       | AlcPINA | Pal5  |
|-------|---------|-------|
| PINA  | MMD     | MMG   | MMG+T |
| PINA expression (percent control) | 1.9  | 35.0  | 320   |

C. [Graph showing average auxin/gemling over time (hours).]
Figure 3

A. 

B. 

C. 

D.
Figure 4

A. Chromosome mitotic index

B. Pal5

C. Hydroxyurea (mM) 0 5 10

D. Glycerol

D. Dextrose

Percent Survival

Percent Survival
A. IP antibody: HA

Blot: Asyc. 43°C Asyc. Benomyl 43°C

HA
PINA

PINA W33A
PINA C126A
WW domain
PI domain

DNA binding domain: NIMA
Activation domain: -Trp,Leu -Trp,Leu,His

PINA
PINA W33A
PINA C126A
WW domain
PI domain

B. DNA binding domain: NIMA
Activation domain: -Trp,Leu -Trp,Leu,His

C. DNA binding domain: -Trp,Leu -Trp,Leu,His

NIMA
NIMA 1-292
NIMA 293-699
NIMA 293-396
NIMA 397-699
Figure 6

| Temperature | Dextrose | Glycerol | Glycerol + Thr |
|-------------|----------|----------|---------------|
| 31°C        | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| 37°C        | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| 41°C        | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |

Legend:
- **pAL5 AlcPinA**
- **pAL5**
- **pAL5 AlcPinA**
- **Wt**
- **nimA5**
- **Wt**
- **nimA5**
- **Wt**
- **nimA5**
PINA is essential for growth and positively influences NIMA function in Aspergillus nidulans

James D. Joseph, Scott N. Daigle and Anthony R. Means

J. Biol. Chem. published online June 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405415200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts