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Evidence for a Mammalian Nim1-like Kinase Pathway Acting at the G0-1/S Transition

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In fission yeast the Nim1 kinase phosphorylates and inactivates the cdc2-inhibitory Wee1 tyrosine kinase. In addition, Nim1 is necessary for an efficient cellular response to nutritional starvation leading to cell cycle arrest in G1. Given the remarkable evolutionary conservation of the cell cycle regulatory mechanism we have investigated the effect of Nim1 expression on the control of the mammalian cell cycle using a plasmid microinjection approach. In synchronised IMR90 human fibroblasts, expression of Nim1 strongly inhibited entry into S-phase. This effect was dependent on the catalytic activity of Nim1 and did not require its regulatory domain. Furthermore we show that co-expression of human Wee1 kinase reverted the inhibitory effect, indicating that Nim1 was acting in a Wee1-dependent manner. These results provide evidence for the existence of a Nim1-like kinase pathway acting at the G0-1/S transition in human cells.

In the fission yeast, *Schizosaccharomyces pombe,* the cdc2 protein kinase plays a central role in the regulation of entry into mitosis. The activity of this enzyme is controlled in response to intra- and extra-cellular signals through a network of regulatory kinases and phosphatases. One of the key events for the maintenance of cdc2 in an inactive state during interphase is the Wee1- and Mik1-dependent phosphorylation of tyrosine 15, a residue located in the ATP-binding region (1-3). At the G2/M transition, activation of cdc2 associated to the cyclin B regulatory subunit is achieved through a cdc25-dependent dephosphorylation of that tyrosine (2, 4). In higher eukaryotes, the cyclin-dependent kinase (CDK1) p34cdc2 is similarly negatively regulated by phosphorylation on both threonine 14 and tyrosine 15 (5-7). Fission yeast and human Wee1 (8, 9) have been shown to phosphorylate human cdc2 on tyrosine 15 (7, 10). Recently, a membrane-associated kinase called Myt1 that is related to the Wee1 kinase was identified in Xenopus and shown to phosphorylate both tyrosine 15 and threonine 14 (11, 12).

The Nim1/cdr1 cell cycle regulator was identified in the fission yeast through two independent experimental approaches. First, Nim1 was cloned on the basis of its ability when overexpressed to rescue a cdc25 thermosensitive defect (13). Second, cdr1 was found to be required for an efficient cellular response to nutritional starvation that allow the exit from the cell cycle in G1 (14-16). Nim1/cdr1 is not an essential gene although Nim1 disruptant cells display an elongated phenotype. On the other hand, cells overexpressing Nim1/cdr1 enter mitosis at a reduced size with a classical 'Wee' phenotype. Together these results indicate that Nim1/cdr1 is an inducer of mitosis (13, 17). Nim1 encodes a 67 kDa protein kinase that has been shown to regulate the activity of the cdc2 protein kinase through the phosphorylation and inactivation of the Wee1 tyrosine kinase (18-21). Nim1 displays highest homology scores with *S. cerevisiae* KKK1/HSL1, a structurally and functionally related kinase (22). It has been shown that HSL1 is a negative regulator of SWE1, the homologue of Wee1 in *S. cerevisae* (22). No functional homologue of the fission yeast Nim1 has been identified so far in vertebrates. However, given the high conservation of the cell cycle machinery between distantly related eukaryotes such as yeast and man, Nim1 would be expected to be also present in other species. In mammals, control of the growth conditions takes place in G1 before cell commitment to a mitotic cycle when entering S-phase (23, 24). Given the role of *S. pombe* Nim1 in connecting the cell cycle machinery to nutritional cues, we speculated that the G1 to S-phase transition in mammals might also involve a Nim1-like kinase activity. We decided to test that hypothesis by examining the effect of ectopic expression of the fission yeast Nim1 kinase on the cell cycle control in human fibroblasts.

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MATERIALS AND METHODS

Cell culture and synchronisation. Human diploid lung fibroblasts (IMR-90), obtained from the American Type Culture Collection (ATCC), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated foetal calf serum (FCS), 2 mM glutamin, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C. IMR90 cells were arrested in G0 by incubating them for 3 days in DMEM without serum. They were stimulated to re-enter into the cell cycle by adding 20% FCS. Progression through the cell cycle was monitored by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation after two hours pulses.

Microinjection procedure and immunofluorescence. Cells growing on glass coverslips were microinjected with 1 fl of a caesium chloride grade plasmids solution (150 μg/ml) containing purified rabbit IgG (2 mg/ml). For each independent experiment an average of 50 cells were injected using an Ependorff micro injector. After BrdU labelling, cells were fixed as described (25) and detection was achieved using the Boerhinger Mannheim detection kit following the manufacturer's instructions. For GFP-Nim1 detection, the cells were fixed in 3.7% formaldehyde at 4°C during 20 min., washed with PBS and the coverslips were mounted in mowiol. Microscopic observations were performed under a Zeiss Axiophot photomicroscope using a Neofluar 100x lens. Photographs were taken on Kodak Tmax 400 film.

Plasmid constructs. pCMV-Nim1 full size (589 residues) and C-terminal truncated (residues 1 to 354) plasmids were constructed by insertion of Nim1 wild type and K41A mutant coding sequences (16) in the pX expression vector (26). pGFP-Nim1C was constructed by insertion of a BamHI-HindIII (klenow) fragment from pCMV-Nim1C into the pGFP-N1 vector (Clontech). pCMV-Wee1 plasmids were constructed by insertion of the StuI-HpaI fragment of Wee1Hu cDNA between the BamH1 and HpaI sites of pSLX-CMV (27), after the StuI site was changed to a BamH1 site using BamH1 linkers.

RESULTS AND DISCUSSION

Nim1 kinase inhibits entry into S-phase. Human primary fibroblasts (IMR90) were cultured in the absence of serum during 3 days and were therefore arrested in a G0 quiescent stage (28). Plasmids encoding various constructions of the Nim1 cDNA under the control of the CMV promoter were then microinjected into the quiescent cells before the addition of 20% foetal calf serum (FCS). As shown in Figure 1A, the expression of a GFP-tagged version of the Nim1 cDNA was detected under fluorescence microscope 24 hours after plasmid microinjection. Nim1 displayed a cytoplasmic localisation similar to what has been described in fission yeast. Stimulated cells were collected at intervals after a two hours labelling with 5-bromodeoxyuridine (BrdU) and the percentage of cells in S-phase was determined by immunofluorescence examination both in the microinjected and in the control neighbouring population. Control non injected cells started to replicate their DNA 15 hours after serum readdition, and by 25 hours about 50 % of the cells were in S-phase (Figure 1B). Most of the cells microinjected with Nim1 expressing plasmid did not enter S-phase and a maximum of 19 % of BrdU positive cells was detected at 25 hours. This inhibition was consistently observed in several other sets of independent experiments (see also Figure 2 and 3). As

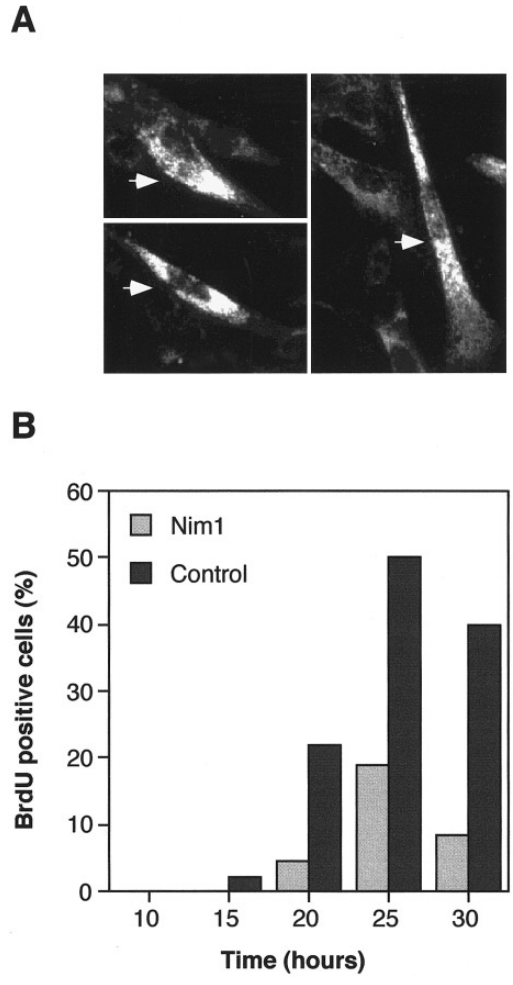
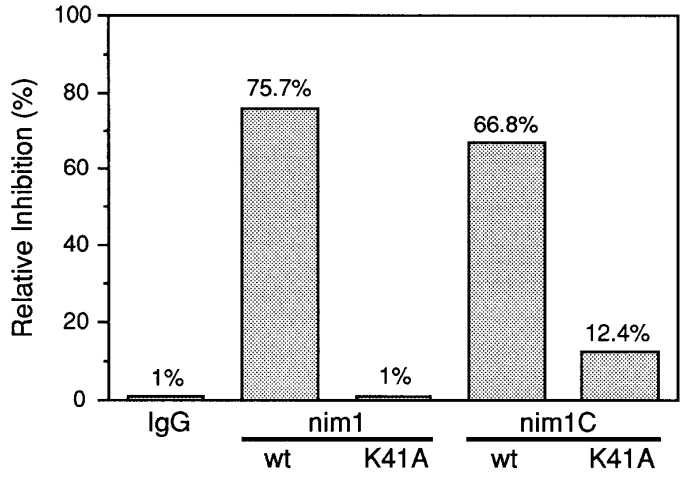


FIG. 1. Microinjection of Nim1 kinase encoding plasmids (pCMV-Nim1) in human quiescent IMR90 diploid fibroblasts inhibits entry into S-phase. G0-arrested cells were obtained by 72 hours serum deprivation and were stimulated by addition of 20% foetal calf serum (FCS). (A) Expression of GFP (green fluorescent protein)-tagged Nim1 was detected 24 hours after plasmid microinjection (pCMV-GFPNim1) and serum addition. Microinjected cells are indicated with a white arrow. (B) Microinjections of Nim1 encoding plasmid (1fl of a 150μg/ml solution) were performed prior to the addition of serum. Cells were co-injected with rabbit IgG, allowing their detection using Texas red-conjugated anti-rabbit antibodies. BrdU was added for a two hour pulse before the indicated fixation time. The graph indicates the percentage of BrdU positive cells among the injected one.

shown in figure 2, the average percentage of cells in S-phase 25 hours after serum readdition was 10.8%, a value that represents about 75% of inhibition. In contrast, microinjection with rabbit IgG had no detectable effect on the timing of entry into S-phase.

Cyclin D1 nuclear accumulation and disappearance as cells proceed into S-phase are essential features of the G1/S transition (28). We did not detect cyclin D1 by immunofluorescence microscopy (data not shown) in Nim1 microinjected cells, indicating that its expression



Injected DNA	Number of indep. Exper.	Injected Cells Analyzed	BrdU in Injected Cells (%)	BrdU in Uninjected Cells (%)	Relative Inhibition (%)
pCMV-nim1	4	165	10.8±2	44.6±2.9	75.7
pCMV-nim1K41A	4	137	42.9±8.3	43.3±8.1	1.0
pCMV-nim1C	3	111	16.3±4.1	49.1±11.1	66.8
pCMV-nim1CK41A	2	51	49.8±4.7	56.9±4.9	12.4

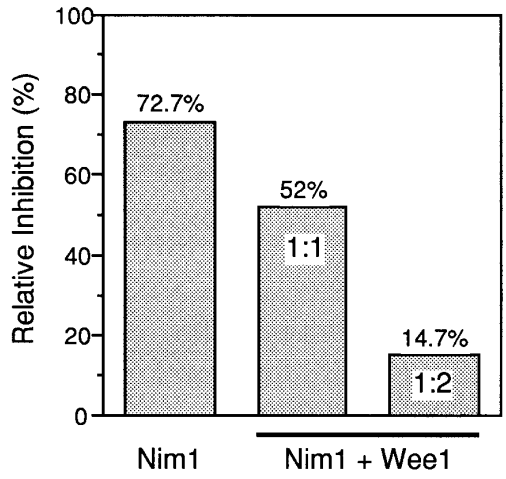
FIG. 2. Nim1 catalytic activity is required for the inhibition of entry into S-phase Human IMR90 fibroblasts, grown for 72 hours in the absence of serum, were microinjected with the indicated plasmids (1fl of a 150μg/ml solution) together with rabbit IgG (2 mg/ml). Twenty-three hours after 20% serum addition BrdU was added. After two hours, cells were fixed and processed for immunofluorescence analysis. Multiple independent experiments were performed as indicated and the average percentage (±SD) of BrdU positive cells was calculated both for injected and uninjected cells. The percentage of relative inhibition of DNA synthesis in injected cells was calculated by the formula: percent = [% of BrdU-positive cells (uninjected) − % of BrdU-positive cells (injected)]/% of BrdU-positive cells (uninjected). These values are the average of independent experiments as indicated.

was either impaired or prevented upon Nim1 induced cell cycle arrest.

Nim1 inhibition of S-phase depends on its kinase activity. Plasmids encoding either active or catalytically inactive K41A Nim1 mutants (both the full length protein or the catalytic domain of Nim1 alone), were microinjected in serum starved G0 arrested cells that were subsequently re-feeded by 20% serum addition. As shown in figure 2, a plasmid encoding only the catalytic domain of the Nim1 kinase (pCMV-Nim1C) largely inhibited entry into S-phase (66.8% inhibition) as full length Nim1 did (75.7% inhibition). In contrast, plasmids encoding inactive K41A mutants, either the full length protein or the catalytic domain alone, did not impair significantly entry into S-phase.

Expression of human Wee1 reverts the Nim1 inhibitory effect. It has been shown *in vitro* that the fission yeast Wee1 protein is a substrate for the Nim1 kinase (18-21). Given the fact that the inhibitory effect of Nim1 was dependent on its catalytic activity, we investigated whether this was acting through Wee1. We therefore performed microinjection of plasmids encoding either the full length human Wee1 kinase alone or in combination with Nim1. On its own, microinjection of the plasmid encoding Wee1Hu had no detectable effect on the entry into S-phase (Figure 3, bottom). When Nim1- and Wee1Hu- encoding plasmids were co-injected, inhibition of entry into S-phase was reverted. This effect was dependent on the ratio between Wee1 and Nim1 plasmids. Although a role for a Wee1-related kinase at that stage of the cell cycle has not been documented, the reversion of Nim1 effect when providing a putative substrate strongly suggested that Nim1 overexpression inhibited re-entry into the cell cycle through a Wee1- or Wee1-like- dependent pathway. Our results therefore suggest that the activity of an as yet unidentified homologue of Wee1 is required for entry into the cell cycle. This activity would be inhibited through the phosphorylation by Nim1 and overexpression of human Wee1 could compensate this effect.

Conclusion. As a step toward the elucidation of the regulation of cyclin-dependent kinases that are in-



Injected DNA	Number of Ind. exp.	Injected Cells Analyzed	BrdU in Injected Cells (%)	BrdU in Uninjected Cells (%)	Relative Inhib. (%)
pCMV-nim1	2	66	11.9±0.9	43.6±0.9	72.7
pCMV-wee1	2	67	43.5±7.8	43±14.1	0.0
nim1 + wee1 (1:1)	1	35	17	36	52.0
nim1 + wee1 (1:2)	3	127	35.3±6.4	41.4±6.5	14.7

FIG. 3. Nim1 effects are reverted by co-injection of Wee1Hu expressing plasmid. IMR90 diploid fibroblasts, grown for 72 hours in the absence of serum, were microinjected with the indicated plasmids and rabbit IgG. Nim1 and Wee1Hu were injected either alone or in combination in a 1:1 or 1:2 ratio, however the total amount of plasmid was kept constant by addition of pCMV empty vector. Twenty-three hours after 20% serum readdition BrdU was added. After two hours cells were fixed and processed for immunofluorescence analysis. Multiple independent experiments were performed as indicated and the average percentage (±SD) of BrdU positive cells was calculated both for injected and uninjected cells. The percentage of relative inhibition of DNA synthesis in injected cells was calculated as indicated in figure 2.

volved in the control of the commitment into the cell cycle, we have investigated the function of a fission yeast cell cycle regulator, namely Nim1, when expressed in human fibroblasts. A similar approach using expression of heterologous protein kinase was successfully used to provide evidence for a NIMA-like Aspergillus mitotic kinase pathway in vertebrate cells (29). We have shown here that the fission yeast Nim1 kinase inhibits entry of human fibroblats into S-phase in a Wee1-like dependent manner.

In fission yeast, the catalytic activity of Nim1 is permanently balancing the Wee1 inhibitory function on cdc2, and Nim1 overexpression induces entry into mitosis prematurely. We did not detect such an effect when we expressed Nim1 in human fibroblasts or in HeLa cells (data not shown). It is therefore likely that others regulatory mechanism that counteract Nim1 effect may also operate in mammals to regulate cdc2 activation. In *S. pombe,* the catalytic activity of Nim1 is also required for an efficient arrest in G0/G1 upon nutritional starvation (16), however the molecular nature of Nim1 involvement at that stage of the cell cycle has not yet been clearly established. Vertebrates cyclin-dependent kinases that are involved in the control of G1/S are also likely to be regulated through the phosphorylation by a Wee1/Mik1-related kinase (30). The inhibition of this pathway could therefore be deleterious for the coordination of the events that are required for the cell cycle progression toward S-phase. Although an human Nim1 homologue has not been yet identified, our results are consistent with the involvement of a Nim1-like pathway in the regulation of the mammalian G0-1/S transition. Experiments are now underway to investigate the physiological significance of these results and to identify Nim1-like upstream regulators of cyclin-dependent kinases in human.

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