Cyclin E-mediated elimination of p27 requires its interaction with the nuclear pore-associated protein mNPAP60

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The Cdk2 inhibitor, p27^{Kip1}, is degraded in a phosphorylation- and ubiquitylation-dependent manner at the G₁–S transition of the cell cycle. Degradation of p27^{Kip1} requires import into the nucleus for phosphorylation by Cdk2. Phosphorylated p27^{Kip1} is thought to be subsequently re-exported and degraded in the cytosol. Using two-hybrid screens, we now show that p27^{Kip1} interacts with a nuclear pore-associated protein, mNPAP60, map the interaction to the 3₁₀ helix of p27 and identify a point mutant in p27^{Kip1} that is deficient for interaction (R90G). *In vivo* and *in vitro*, the loss-of-interaction mutant is poorly transported into the nucleus, while ubiquitylation of p27^{R90G} occurs normally. *In vivo*, co-expression of cyclin E and Cdk2 rescues the import defect. However, mutant p27^{Kip1} accumulates in a phosphorylated form in the nucleus and is not efficiently degraded, arguing that at least one step in the degradation of phosphorylated p27^{Kip1} requires an interaction with the nuclear pore. Our results identify a novel component involved in p27^{Kip1} degradation and suggest that degradation of p27^{Kip1} is tightly linked to its intracellular transport.

Keywords: degradation/intracellular transport/mNPAP60/p27^{Kip1}

Introduction

The p27^{Kip1} protein has a dual role in the G₁ phase of the cell cycle: it inhibits Cdk2 complexes in resting cells and during the G₁ phase of the cell cycle, and promotes assembly and nuclear import of cyclin D–Cdk4 complexes (for review, see Sherr and Roberts, 1999). At the end of the G₁ phase, p27^{Kip1} is degraded. Degradation of p27^{Kip1} is sensitive to inhibitors of proteasome function, suggesting that degradation occurs via the ubiquitin pathway (Pagano *et al.*, 1995). Ubiquitylated forms of p27^{Kip1} have been detected *in vivo* and degradation and ubiquitylation have been reconstituted *in vitro* using partially purified systems (Montagnoli *et al.*, 1999; Nguyen *et al.*, 1999; Shirane *et al.*, 1999).

Two mechanisms are known that ensure the correct timing of p27^{Kip1} degradation. First, p27^{Kip1} is a substrate of cyclin E–Cdk2 kinase and is phosphorylated by Cdk2

mainly, but not exclusively, at Thr187 (Müller *et al.*, 1997; Sheaff *et al.*, 1997; Vlach *et al.*, 1997). *In vivo*, degradation of p27^{Kip1} requires active Cdk2 kinase and mutation of Thr187 inhibits degradation; *in vitro*, phosphorylation of Thr187 is required for ubiquitylation and subsequent degradation of p27^{Kip1} (Montagnoli *et al.*, 1999; Tsvetkov *et al.*, 1999). Together, the findings show that active cyclin–Cdk2 complexes during the G₁ phase are required for elimination of p27^{Kip1}.

Secondly, skp2 has been identified as a candidate component of the E3 ligase complex that ubiquitylates p27^{Kip1} (Carrano *et al.*, 1999). *In vivo*, skp2 function is required for p27^{Kip1} degradation (Carrano *et al.*, 1999). Ectopic expression of skp2 in quiescent cells stimulates entry into S phase of the cell cycle and promotes degradation of p27^{Kip1}, providing evidence that the amount of skp2 is rate limiting for degradation of p27^{Kip1} (Sutterlüty *et al.*, 1999). In normal cells, expression of skp2 is restricted to the S phase (Zhang *et al.*, 1995; Lisztwan *et al.*, 1998). Although the mechanisms underlying this restriction are not known, the findings suggest that regulation of skp2 expression is a second mechanism that contributes to the correct timing of p27^{Kip1} degradation.

In order to exert its function during G₁ and to be degraded at the end of G₁, p27^{Kip1} needs to be imported into the nucleus (Reynisdottir and Massague, 1997; Tomoda *et al.*, 1999). Phosphorylation of p27^{Kip1} by Cdk2 is restricted to the cell nucleus due to the nuclear localization of CAK (Darbon *et al.*, 1994), which may account for the requirement for nuclear import in degradation. A nuclear localization signal (NLS) has been assumed to be responsible for transport of p27^{Kip1} into the nucleus, since deletion of the C-terminus of p27^{Kip1} including the NLS abolishes import (Reynisdottir and Massague, 1997; Tomoda *et al.*, 1999). Recently, a component of the signalosome, Jab1, has been found to interact with p27^{Kip1} and promote its degradation. Jab1 interacts with p27^{Kip1} and promotes its export from the nucleus, suggesting that degradation of phosphorylated p27^{Kip1} occurs in the cytosol (Tomoda *et al.*, 1999). The signals mediating nuclear export of p27^{Kip1} are unknown.

Using two-hybrid cloning we have identified an interaction between p27^{Kip1} and a nuclear pore-associated protein and provide evidence that this interaction is required for nuclear import of p27^{Kip1} and for degradation of phosphorylated p27^{Kip1} after nuclear import. Our findings identify a new component involved in p27^{Kip1} metabolism, support the notion that degradation of phosphorylated p27^{Kip1} requires export from the nucleus and have implications for the mechanisms by which intracellular transport of p27^{Kip1} is regulated.

Results

In order to identify novel proteins that interact with p27, we screened a mouse embryo cDNA library using full-

length mouse p27 as bait. Of 500 positive clones, all except two coded for known cyclins (not shown); the remaining two were identical and encoded a novel p27-interacting protein (Figure 1A). The longest cDNAs that

were isolated from a Balb/c-3T3 library encode a protein of 466 amino acids; the fragment recovered in the two-hybrid screen corresponds to amino acids 121–252 of the full-length protein. Western blotting using affinity-purified antibodies raised against this protein reveals an endogenous protein in a number of different cell lines; the endogenous protein comigrates with a protein expressed from a cDNA clone by transient transfection, demonstrating that a full-length cDNA was isolated (Figure 1B). Immunofluorescence experiments using these antibodies detected the endogenous protein in a speckled pattern at the nuclear periphery highly reminiscent of nuclear pore proteins or nuclear transport factors; mNPAP60 expressed by transient transfection showed a very similar distribution (Figure 1C). Database searches revealed that a rat cDNA homologous to the clones we had isolated had been described previously; the rat cDNA encodes a nucleoporin termed NPAP60 (Fan *et al.*, 1997). We therefore refer to our clone as m(ouse)NPAP60. Both clones differ by a small sequence divergence in the N-terminus and by the presence of a putative RAN-binding domain in the C-terminus of the clone we have recovered, suggesting that the rat clone may possibly represent a shorter splice variant (see Supplementary figure 1, available at *The EMBO Journal* Online). Weak similarities to other nucleoporins were found, mainly within the putative RAN-binding domain.

A number of controls established that mNPAP60 interacts specifically with p27 in the yeast two-hybrid assay (Figure 1A). In order to verify that p27 and mNPAP60 interact outside the context of two-hybrid experiments, we generated and purified a glutathione *S*-transferase (GST)–mNPAP60 chimeric protein and incubated it with ³⁵S-labelled p27 translated in a reticulocyte lysate (Figure 1D). In these experiments, p27 interacted efficiently with GST–mNPAP60, but not with equal amounts of GST alone. In similar experiments, no specific interaction of mNPAP60 was seen with cyclin D or E or with p21^{Waf1} (data not shown). p27 present in lysates of RAT1-MycER cells under conditions where it is bound to Cdk2 and Cdk4 (as judged by immunoprecipitation and gel filtration; not shown) did not interact with GST–mNPAP60. In contrast, p27 extracted from such lysates by a brief heat treatment specifically bound to

GST–mNPAP60, suggesting that binding of p27 to Cdk2 and Cdk4 complexes prevents interaction with mNPAP60 *in vitro* (Figure 1E). Consistent with this notion, pre-incubation of p27 with cyclin E–Cdk2 complexes purified from insect cells infected with recombinant baculovirus abolished the interaction with GST–mNPAP60 (Figure 1D).

An interaction between mNPAP60 and p27 was also detected by immunoprecipitation from cellular lysates (Figure 1F). HeLa cells were transfected with CMV-based expression vectors encoding p27 and mNPAP60 and detergent lysates were precipitated with either anti-mNPAP60 antibodies or preimmune antiserum. In these experiments, p27 was detected in anti-mNPAP60 immunoprecipitates when both proteins were co-expressed. No p27 was detected in precipitates using preimmune serum; also, p27 was not detected in anti-mNPAP60 immunoprecipitates from cells in which mNPAP60 had not been expressed. We noticed that expression of large amounts of mNPAP60 diminished expression of p27; addition of the proteasome inhibitor LLNL partly reversed this effect (not shown). Close inspection of the gels suggested that mNPAP60 associated preferentially with a slower-migrating, phosphorylated form of p27 (not shown). Therefore, we repeated the experiment with a mutant of p27 that lacks the Cdk2 phosphorylation site (T187V). In contrast to the two-hybrid situation (see below), this mutation diminished the association of p27 with mNPAP60 (Figure 1F). Potentially, therefore, proteins that specifically recognize the phosphorylated form of p27 stabilize the p27–mNPAP60 complex *in vivo*.

The antibodies we have raised do not detect the human mNPAP60 protein. To determine whether endogenous mNPAP interacts with p27, lysates of NIH 3T3 cells were immunoprecipitated with anti-p27 and control antibodies. In the precipitates, mNPAP60 could be detected in anti-p27, but not in control precipitates, demonstrating that the endogenous proteins associate with each other (Figure 1G).

To map the interaction surface between p27 and mNPAP60, progressive N- and C-terminal deletions of p27 were tested in the two-hybrid assay and by *in vitro* pull-down experiments (Figure 2A). Both assays gave consistent results, which map the N-terminal border of the interaction domain between amino acids 69 and 91 of p27

Fig. 1. Identification of mNPAP60 as a p27-interacting protein. (A) Summary of yeast two-hybrid interaction data. Plasmids expressing the indicated chimeras were transformed into yeast strains and β -galactosidase activity was determined by a semi-quantitative filter assay. (B) Western blot documenting the expression of endogenous mNPAP60 in rodent fibroblast cell lines (left lanes). The right lanes contain extracts from HeLa cells transfected with vector alone or with an expression vector encoding mNPAP60. The antibody raised against mouse NPAP does not cross-react with human NPAP. (C) Immunofluorescence of cultured RAT1 cells using an affinity-purified antiserum raised against mNPAP60 (left). The right panel shows the localization of mNPAP60 expressed by transient transfection in HeLa cells. The middle panel shows a control after pre-incubation of the antiserum with a GST–mNPAP60 fusion protein. The pictures are a false-colour superimposition of the 4',6-diamidino-2-phenylindole-stained nuclei (coloured in blue) and anti-mNPAP60 fluorescence (coloured in red). (D) *In vitro* binding of ³⁵S-labelled p27 to GST–mNPAP60(121–252). The upper panel shows Coomassie-stained gels of the input into the *in vitro* binding reactions together with a molecular weight marker. BSA was added to reduce background binding. The middle panel shows a fluorography demonstrating specific binding of p27 to GST–mNPAP60. The lower panel shows reduced binding of p27 to GST–mNPAP60 after pre-incubation of p27 with cyclin E–Cdk2 complexes purified from baculovirus. The input lanes correspond to 10% of the total amount of p27 loaded on to the GST beads. (E) *In vitro* binding reactions from cell lysates. Detergent lysates from RAT1-MycER cells were prepared with or without heat treatment and incubated with GST–mNPAP60 as above. Western blots of the input lanes (10% of loading) and the recovered beads are shown. (F) Co-immunoprecipitation of p27 with anti-mNPAP60 antibodies. HeLa cells were transfected with the expression plasmids indicated, lysates prepared and immunoprecipitated with either antiserum against mNPAP60 or preimmune serum. Shown are Western blots probed with the antibodies indicated. N.S. reflects a non-specific band present in all immunoprecipitates. mNPAP60 migrates just above the IgG heavy chain on SDS gels; since the same antibody is used for both immunoprecipitation and Western blotting, the heavy chain is visible below the mNPAP60 bands in the immunoprecipitations. (G) Co-immunoprecipitation of endogenous mNPAP60 with anti-p27, but not with control antibodies from lysates of NIH 3T3 cells. The input represents 5% of cell extract used for the precipitation.

A **B** **C** **D**

Fig. 2. Isolation of a mNPAP60 loss-of-interaction mutant of p27. (A) Summary of two-hybrid data (left) and *in vitro* interaction assays (right) documenting the interaction of C-terminal, N-terminal and internal deletion mutants of p27 with mNPAP60. The assays were carried out as described in the legend to Figure 1. p27 Δ 91-197 lacks three of five methionines and is, therefore, labelled more weakly in these experiments. The right panel also documents the reduced interaction of the p27R90G mutant with GST-mNPAP60 *in vitro*. (B) Western blot documenting co-immunoprecipitation of p27wt, but not of p27R90G after co-expression with mNPAP60 in HeLa cells. Assays were carried out as described in the legend to Figure 1. (C) Inhibition of cyclin E-Cdk2 kinase activity by increasing amounts of p27. His-tagged recombinant p27wt and p27R90G proteins were purified and incubated with cyclin E-Cdk2 complexes isolated from baculovirus lysates. Histone H1 was used as a substrate. Triplicate assays are shown and were used to calculate the K_i values shown on the right. (D) The proteins indicated were purified and equal amounts subjected to phosphorylation by immunoprecipitated cyclin E-Cdk2 kinase. Shown is an autoradiogram of triplicate assays. The control designated '-cE/Cdk2' contains p27wt protein and a mock precipitate using no antibody.

and the C-terminal border between amino acids 85 and 94. An internal deletion mutant of p27 spanning amino acids 85-98 failed to interact with mNPAP60 both *in vitro* and in two-hybrid assays (Figure 2A). Thus, the interaction surface centres on the 3₁₀ helix of p27, overlapping the cdk2-binding domain of the protein. This is consistent with the *in vitro* observations demonstrating that cdk2 and mNPAP compete for binding to p27.

In order to identify a mutant of p27 that specifically disrupts interaction with mNPAP60, a loss-of-interaction screen was performed in yeast. p27 was mutagenized using mismatch PCR in the presence of manganese ions and transformed in *Escherichia coli*. A total of 950 colonies were picked and the plasmids isolated were tested in a two-hybrid reaction with mNPAP60; 179 candidate negative colonies were selected, the plasmid isolated and re-

Fig. 3. Localization of p27 mutants in HeLa cells. (A) Quantitation of the results of several independent transfections into HeLa cells. (B) Examples of individual transfections.

transformed. From these, 22 plasmids were isolated, which encoded p27 proteins that failed to interact with mNPAP60. The plasmids were retested in a two-hybrid assay using cyclin D1 as prey and the p27 open reading frames of these plasmids were sequenced. Three plasmids were recovered that encoded mutant p27 proteins that interacted with cyclin D1 but failed to interact with mNPAP60 (see Supplementary figure 2). All three carried a single point mutation at position 90 (R90G). The R90G mutation was not present in any of the other plasmids recovered.

In this screen, the mutations we recovered were exclusively A to G transitions (not shown). Therefore, the specificity of the arginine (AGG) to glycine (GGG) change is almost certainly due to the mutagenesis protocol we used. In wild-type p27, the arginine residue that is affected by the mutation forms a salt bridge with a glutamic acid residue at position 85; thus, it is very likely

that this mutation at position 90 will affect the stability of the 3₁₀ helix (Russo *et al.*, 1996). For all subsequent experiments, this mutation was re-introduced in a wild-type p27 plasmid and the plasmid was sequenced to ensure that it carries this single mutation. The localization of the mutation relative to other mapped interactions in the p27 sequence and within the p27 crystal structure is shown in Supplementary figure 2.

To test whether the mutation indeed specifically disrupts the interaction with mNPAP60, we used *in vitro* pull-down experiments as before: in these experiments, p27R90G failed to interact with GST-mNPAP60 (Figure 2A). Also, p27R90G expressed by transient transfection in HeLa cells together with mNPAP60 failed to be precipitated by antibodies directed against mNPAP, in contrast to wild-type p27 (Figure 2B). We concluded that the R90G mutation disrupts association of p27 with mNPAP60 both *in vitro* and *in vivo*.

Fig. 4. Characterization of the nuclear import of p27. (A) Recombinant p27wt and p27R90G proteins were fluorescently labelled and injected into the cytosol of exponentially growing HeLa cells. The time course of import is shown; between 50 and 150 cells were injected and evaluated for each time point. (B) Example of *in vitro* import reactions using fluorescently labelled p27 proteins and permeabilized HeLa cells. (C) Summary of results of *in vitro* import reactions.

To test whether the mutation affects interaction with cyclin E-Cdk2 complexes, p27 and p27R90G were expressed in *E. coli* as his-tagged recombinant proteins and incubated with cyclin E-Cdk2 complexes isolated from baculovirus-infected cells. In these assays, both proteins were indistinguishable. They inhibited cyclin E-Cdk2 kinase activity with identical K_i values and cyclin E-Cdk2 efficiently phosphorylated both proteins *in vitro* (Figure 2C and D). This result is in agreement with data obtained for other mutations in the 3₁₀ helix (Hashimoto *et al.*, 1998).

To investigate whether mNPAP60 is involved in the intracellular transport of p27, we expressed wild-type p27

and p27R90G by transient transfection in a number of cell lines and obtained similar results in HeLa, MCF7 and RAT1 cells. The results obtained in HeLa cells are summarized in Figure 3A. Examples of individual transfections are shown in Figure 3B. Using equal amounts of expression plasmid, a significant proportion of cells expressing p27R90G, but not p27wt, showed strong cytosolic staining in addition to nuclear staining. Similarly, the internal deletion mutant p27Δ85–98 mislocalized to the cytosol in a large fraction of the cells.

To determine the relevance of the NLS of p27 for nuclear transport in detail, the signal was disrupted by the replacement of three basic amino acids that constitute part

Fig. 5. Inhibition of cell proliferation by p27wt and p27R90G. RAT1 cells were infected with recombinant retroviruses expressing either p27wt or p27R90G. (A) The rate of cell proliferation of resistant cell pools relative to a pool of cells infected with a control (pbabe-puro) virus. (B) Average doubling time calculated from logarithmic plots of two independent infections. (C) Western blots documenting the expression of the proteins indicated and the amounts of cyclin A/p27 and cyclin E/p27 in exponentially growing cells infected with the viruses indicated.

of the NLS (R152A K153E R154A). This mutant showed a partial relocalization to the cytosol. A double mutant of p27 that carries both a mutation of the NLS and the R90G mutation mislocalizes to the cytosol in almost all of the transfected cells. The consistent mislocalization of both the R90G and the p27 Δ 85–98 mutations suggests a role for the mNPAP60–p27 interaction in the nuclear import of p27.

To confirm this suggestion, recombinant p27wt and p27R90G proteins were fluorescently labelled and equal amounts of protein were injected into either the cytosol or the nucleus of HeLa cells (Figure 4A). When injected into the cytosol, wild-type p27 was rapidly imported into the cell nucleus; half-maximal import was observed 5 min after injection. In contrast, p27R90G was transported more slowly into the nucleus; in this assay the rate of import was ~2-fold lower than that of wild-type p27. The data support the notion that the mNPAP60–p27 interaction has a functional role in nuclear import. When injected into the nucleus of HeLa cells, neither p27wt nor p27R90G were exported within the time frame of the experiment to a detectable degree; instead, the observable fluorescence decayed over the next 30 min. The data suggest that both proteins are retained in the nucleus. We could not, therefore, establish from these experiments whether mNPAP60 has a role in the nuclear export of p27 (see below).

To characterize the mechanism of import in more detail, we tested both p27wt and p27R90G using *in vitro* nuclear import assays. An example of the results is shown in Figure 4B, and the results are summarized in Figure 4C. Wild-type p27 was imported efficiently into the nucleus in these assays. Import was strictly dependent on temperature and was abolished at 0°C; import was also abolished by addition of either wheat-germ agglutinin or a dominant-negative fragment of importin- β known to disrupt nucleoporin function (Kutay *et al.*, 1997). Thus, nuclear import of p27 relies on a functional interaction with the nuclear pore and does not occur by free diffusion. This is expected, as recombinant p27 forms a dimer with an apparent molecular weight of 70 kDa in gel filtration assays (D.Müller and M.Eilers, unpublished data). Depletion of ATP, depletion of cytosol, addition of the importin- β -binding domain of importin- α (Klebe *et al.*, 1995), addition of non-hydrolysable analogues of GTP or addition of a dominant-negative mutant of Ran (Weis *et al.*, 1996) did not inhibit import. In parallel experiments, these treatments inhibited import of an NLS–bovine serum albumin (BSA) conjugate, as expected (not shown). Thus, nuclear import of p27 does not depend on the recognition of the NLS by importin- α or an energy-dependent mechanism. Import of p27R90G was strongly diminished relative to wild-type p27 under all conditions tested. We concluded that the *in vitro* nuclear import of p27 occurs via

Fig. 6. Cyclin E–Cdk2-induced degradation requires interaction of p27 with mNPAP60. HeLa cells were transfected with the indicated expression plasmids and analysed by Western blotting. (A) Control experiment documenting expression of cyclin E, Cdk2 and p27wt after transfection of the indicated amounts of plasmid; 5 μ g of CMV–cyclin E and CMV–Cdk2 were used. (B) Repeat of the experiment using the indicated alleles of p27. Double lines indicate the presence of a phosphorylated form of p27; single arrows indicate absence of phosphorylated p27. (C) Cycloheximide-chase experiment documenting enhanced stability of phosphorylated p27R90G relative to p27wt. Transfections were carried out as in (B). The lower panels show a quantitation of the results and the upper panels show Western blots from a representative experiment. (D) p27R90G accumulates in the nucleus upon expression of cyclin E and Cdk2. Shown are immunofluorescence pictures of HeLa cells transfected with the expression vectors indicated together with a quantitation of the experiment.

a process of facilitated diffusion across the pore and that interaction of p27 with mNPAP60 is required for this process.

Upon expression from retroviral vectors, p27 inhibits proliferation of RAT1 cells (Vlach *et al.*, 1996; Müller *et al.*, 1997). To determine whether the interaction with mNPAP60 affects this function of p27 *in vivo*, we constructed retroviral vectors that express either wild-type or mutant p27 together with a puromycin resistance gene. RAT1 cells were infected with retroviral supernatants and pools of resistant cells selected; these pools were plated at low density to determine the rate of proliferation of these cells. Surprisingly, p27R90G consistently affected the rate of proliferation more strongly than wild-type p27 in repeated experiments (Figure 5A and B).

To explain this finding, we considered the possibility that ectopic expression of p27R90G might indirectly cause mislocalization of cyclins that lack an NLS such as cyclin A, but immunofluorescence experiments showed that this was not the case (not shown). However, in exponentially growing cells expressing p27R90G, levels of p27 were elevated relative to p27wt and elevated levels of p27R90G bound to cyclin E compared with either uninfected cells or cells infected with a virus expressing p27wt, indicating a defect in cyclin E-mediated elimination of p27 (Figure 5C).

To confirm this suggestion, we co-expressed cyclin E and Cdk2 with different forms of p27 by transient transfection of HeLa cells (Figure 6A and B). In these assays, expression of cyclin E and Cdk2 induced efficient elimination of wild-type p27. This effect on p27 was fully reversible by addition of LLNL, an inhibitor of proteasomal degradation (not shown). In contrast, p27R90G was significantly more stable than wild-type p27; indeed, mutation at R90 stabilized p27 to a similar degree to a mutation of the major Cdk2 phosphorylation site, Thr187. Combining both mutations showed a strong synergy and yielded a mutant of p27 that is resistant to cyclin E-mediated elimination (Figure 6B).

To show that the enhanced levels of p27R90G observed after expression of cyclin E–Cdk2 were due to a decreased degradation of the protein, cycloheximide-chase experiments were performed. Cycloheximide was added to a number of dishes 48 h after transfection, cells were harvested after different time points and the amount of remaining p27 was determined by Western blotting (Figure 6C). In these experiments, p27R90G was significantly more stable than p27wt. In particular, mutation at R90 stabilized the phosphorylated form of p27 relative to p27wt; in contrast, the mutation had little effect on the stability of non-phosphorylated p27, which is also degraded in these assays (albeit at a slower rate). From several such experiments, we calculated $t_{1/2}$ s of 60 min and of 240 min for the phosphorylated form of p27wt and p27R90G, respectively, the double mutant (T187VR90G) was degraded with a $t_{1/2}$ of 250 min (not shown). Taken together, the data show that mutation of R90 inhibits degradation of phosphorylated p27.

Since the p27R90G that accumulated upon co-expression of cyclin E and Cdk2 was phosphorylated (see arrows) and since cyclin E–Cdk2 complexes are only active in the cell nucleus (due to nuclear localization of CAK), the

findings show that under these conditions interaction with mNPAP60 is required at a step after nuclear import. Indeed, the remaining fraction of p27R90G accumulated in the nucleus when co-expressed with cyclin E–Cdk2 (Figure 6D). We suggest that the NLSs present in cyclin E mediate efficient nuclear import of a ternary cyclin E–Cdk2–p27R90G complex in these assays.

The failure of p27R90G to be correctly degraded might be a consequence of its defects in intracellular transport or may reflect a direct role of mNPAP60 in ubiquitylation of p27. To discriminate between the two possibilities, we performed *in vitro* ubiquitylation/degradation assays (Figure 7). Consistent with previous reports, wild-type p27, but not p27T187V, was efficiently ubiquitylated in extracts from S phase HeLa cells, confirming that phosphorylation by cyclin E–Cdk2 is required for ubiquitylation (Montagnoli *et al.*, 1999; Nguyen *et al.*, 1999). In contrast, mutation of Arg90 had no effect in these assays; as expected, the double mutant (p27R90G T187V) was not ubiquitylated. The findings show that interaction with mNPAP60 is not required for ubiquitylation of p27 *in vitro*.

To provide an independent confirmation that interaction of p27 with mNPAP60 is required for efficient degradation, we sought to determine whether fragments of the protein act as dominant-negative alleles that inhibit cyclin E–Cdk2-mediated degradation of p27. The protein was arbitrarily divided into an N-terminus, a central p27-interacting domain and the C-terminus that includes the putative RAN-binding domain (Figure 8A). The individual fragments were tagged and co-expressed with cyclin E–Cdk2 and wild-type p27 to test whether degradation of p27 is affected. Western blots using an anti-mNPAP antibody documented that all fragments that are detectable by the mNPAP antibody were expressed in comparable amounts (see Supplementary figure 3); expression of the remaining fragments were detectable using an anti-MycTag antibody (Figure 8B).

In these experiments, expression of full-length mNPAP60 further diminished expression of p27. In contrast, expression of the N-terminal fragment of mNPAP60 inhibited cyclin E–Cdk2-mediated degradation of p27; expression of the p27-binding domain gave a weak inhibition. Neither protein affected the expression of either cyclin E or Cdk2. Cycloheximide-chase experiments showed that the enhanced levels of p27 were due to enhanced stability of the protein in the presence of the N-terminal domain (Figure 8B). Expression of the putative RAN-binding domain gave inconsistent results (Figure 8A). In cells expressing the N-terminal fragment of mNPAP60, p27 mislocalized to the cytosol in transfected cells (Figure 8C). In contrast, the nuclear localization of Myc was not affected in these cells (Figure 8C).

These results provide independent confirmation that mNPAP60 specifically affects intracellular transport of p27 and suggest that the N-terminus of mNPAP60 interacts with additional proteins involved in either transport or degradation of p27.

Discussion

In this work, we describe an interaction of p27 with a nuclear pore-associated protein, mNPAP60 (Fan *et al.*,

Fig. 7. *In vitro* ubiquitylation of p27 does not require interaction with mNPAP60. Shown are fluorograms of *in vitro* ubiquitylation reactions using p27 synthesized in a reticulocyte lysate and an S phase extract from HeLa cells. The panel shows time-courses of ubiquitylation reactions using the indicated alleles of p27. Controls established that the bands indicated are generated in a ubiquitin- and ATP-dependent manner (not shown). The * indicates a background band that is present in reactions in the absence of ubiquitin and HeLa extracts.

1997; Trichet *et al.*, 1999). From the phenotype of a loss-of-interaction mutant of p27 and the phenotype of cells overexpressing a dominant-negative allele of mNPAP60 we conclude that the interaction between p27 and mNPAP60 is required for correct intracellular transport and degradation of p27.

Based on the phenotype of progressive C-terminal deletion mutants of p27, its nuclear import was thought to depend exclusively on the integrity of a bipartite nuclear import signal located around amino acid 153 (Reynisdottir and Massague, 1997). However, a triple point mutant that disrupts the NLS only partially abolishes nuclear import of p27 *in vivo*. In a direct comparison, this phenotype differs strikingly from a C-terminal deletion mutant that removes amino acids 91–197 (D.Müller, unpublished). Therefore, deletion of the conserved C-terminus removes additional elements that directly or indirectly influence the apparent nuclear localization of p27.

Mutation of R90 abolishes the residual nuclear import of p27 that carries a disrupted NLS, arguing that it is mediated by interaction with mNPAP60. A similar use of two distinct nuclear transport pathways by a single protein has been observed for MAP kinase, where monomeric and dimeric forms of the protein are imported via distinct transport routes (Adachi *et al.*, 1999). Similarly, several observations support a model in which p27 bound to Cdks is transported via an NLS-dependent mechanism whereas free p27 (or bound to other partner proteins) is preferentially transported via association with mNPAP60. For example, Cdk2 (and potentially Cdk4) competes for binding of p27 to mNPAP60 *in vitro* most likely because the 3₁₀ helix is part of the Cdk2-interacting surface of p27 (Russo *et al.*, 1996). *In vivo*, the import defect of the R90G mutant is most pronounced under conditions where p27 is in excess of cyclin–Cdk complexes (see, for example, Figure 6C). The model would predict that the NLS is masked in free p27, potentially due to dimerization of the protein; this is consistent with the finding that recombinant p27R90G is not imported via an NLS-dependent mechanism *in vitro*.

In vivo, p27R90G is a more potent inhibitor of proliferation than wild-type p27. Initially, we thought

that the defect in nuclear import accounted for this phenotype. For example, p27R90G might associate in the cytosol with cyclins that lack a nuclear import signal such as cyclin A. Indeed, transiently transfected cells that express large amounts of p27R90G showed a moderate mislocalization of cyclin A to the cytosol. However, this phenotype required high levels of p27 and was not observed in retrovirally infected cells (D.Müller, unpublished data).

Instead, several observations suggest that a defect in cyclin E–Cdk2-mediated elimination of p27 accounts for the more potent inhibition of cell proliferation by p27R90G. First, elevated levels of cyclin E are found associated with p27R90G relative to p27wt in retrovirally infected cells. Secondly, mutation at R90 renders p27 more resistant to cyclin E–Cdk2-induced degradation in transient transfection assays; the mutation synergizes with a mutation in the major Cdk2 phosphorylation site. p27R90G accumulates in the nucleus when co-expressed with cyclin E (which contains an NLS), providing evidence that the interaction between p27 and mNPAP60 is required for degradation of p27 at a step after import into the nucleus.

This requirement does not reflect a direct role for mNPAP60 in ubiquitylation of p27, since p27R90G was efficiently ubiquitylated *in vitro*. The phenotype of the mutant is, therefore, compatible with two models: first, essential components involved in p27 degradation may be localized at the nuclear pore. In this view, interaction of p27 with mNPAP60 is required to target p27 efficiently to components of the degradation pathway. For example, it has been shown that proteasomes accumulate at the nuclear periphery (Enenkel *et al.*, 1998; Wilkinson *et al.*, 1998).

Alternatively, the phenotype of the mutant reflects a requirement for export of phosphorylated p27 from the nucleus for degradation. Very similar models have been proposed for p53, which is exported by association with mdm2 for degradation in the cytosol (Freedman and Levine, 1998). Indeed, the enhanced inhibition of proliferation by p27R90G is reminiscent of p53 in the presence of p19ARF (Tao and Levine, 1999) or of inhibitors of

Fig. 8. The N-terminus of mNPAP60 acts as a dominant-negative inhibitor of cyclin E-Cdk2-mediated degradation of p27. **(A)** Diagram showing the constructs used and their effect on cyclin E-Cdk2-mediated degradation of p27wt (left). The right panel documents the expression of individual fragments of mNPAP60 using α -MYCtag antibodies. Assays were performed essentially as in Figure 6A. **(B)** Left panels show Western blots documenting expression of p27wt, cyclin E and Cdk2 after transient transfection into HeLa cells together with the expression plasmids indicated. LLNL was added to the cells 8 h before harvesting. Cdk4 was used as a control for equal loading. The right panels document the altered stability of p27wt in the presence of full-length mNPAP or in the presence of the N-terminal (1–121) fragment using a cycloheximide-chase experiment. **(C)** Immunofluorescence pictures documenting the subcellular localization of p27wt and Myc in the absence and presence of co-transfected mNPAP60(1–121) in HeLa cells.

nuclear export (Freedman and Levine, 1998), which induce the accumulation of non-degradable, biologically active p53 in the cell nucleus. Potentially, therefore, there is a general requirement for nuclear export in degradation of negative regulators of cell proliferation (Scheffner, 1999).

Nuclear p27 interacts with Jab1, a component of the signalosome (Tomoda *et al.*, 1999). Ectopic expression of

Jab1 has been suggested to promote cell proliferation by facilitating nuclear export and degradation of p27. Jab1 and mNPAP60 may, therefore, act either sequentially in a single pathway or in parallel pathways directing export of p27 from the nucleus. In our experiments, ectopic expression of Jab1 reduced protein levels of both nuclear and cytosolic p27 to almost undetectable levels, precluding an analysis of Jab1-mediated nuclear export by

transient transfection experiments (D.Müller, unpublished data). A detailed analysis of the individual events following phosphorylation of p27 in the nucleus using *in vitro* export assays will be required to resolve these open issues.

Materials and methods

DNA manipulation

For two-hybrid cloning, the entire coding region of mouse p27 was amplified and inserted into pGBT10 (Clontech). A total of 10⁵ independent transformants of a mouse embryonal cDNA library (kind gift of S.Hollenberg) were screened. The loss-of-interaction screen was performed according to Hateboer *et al.* (1995). Full-length mNPAP60 clones were isolated from a λ ZAP Balb/c-3T3 cDNA library, rescued into pBluescript by phage excision and recloned into pcDNA3 for expression. Details of the generation and sequence of individual p27 mutants will be provided upon request; the sequence of all mutants was verified by sequencing. For retroviral expression, p27 cDNAs were inserted into pbabe-puro (Morgenstern and Land, 1990). The sequence of mouse mNPAP has been submitted to the GenBank and EMBL databases (accession No. AF251799).

Cell culture

HeLa, RAT1, MCF7 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium without phenol red supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. Where indicated, 5 μ g/ml puromycin was added for selection and 2.5 μ g/ml for sustained expression of viral genes. Transient transfection into HeLa cells was performed by a calcium phosphate precipitation protocol as described earlier (Desbarats *et al.*, 1996). Retroviral supernatants were generated by transient transfections of BOS23 or Phoenix^{ECO} cells (Pear *et al.*, 1993; Grignani *et al.*, 1998) and used to infect RAT1 cells as described (Bouchard *et al.*, 1999). LLNL (Sigma) was used at a final concentration of 100 μ M and cells were incubated for 8 h in the presence of the chemical.

Protein analysis

In vitro cyclin E-Cdk2 kinase assays and heat treatment of p27 have been described previously (Müller *et al.*, 1997). *In vitro* ubiquitylation assays were carried out as described (Montagnoli *et al.*, 1999). Polyclonal antibodies against cdk2 (M2), cMyc (N262), cdk4 (C22) and monoclonal antibody against human cyclin E (HE-12) were obtained from Santa Cruz Biotechnology. Monoclonal antibody anti-p27(K25020) was obtained from Transduction Laboratories. A polyclonal antiserum against mNPAP60 was raised by immunizing rabbits with a his-tagged fragment of mNPAP60 (amino acids 121–252) expressed in *E.coli*. For *in vitro* pull-down experiments, a fragment encoding amino acids 121–252 of mNPAP was expressed as a fusion protein with GST. Immunofluorescence microscopy was performed as described previously (Peukert *et al.*, 1997) except that cell samples were analysed on a Leica DMIRB microscope and pictures were taken with a Kappa CF 8/1 FMC CCD camera and electronically processed. A minimum of 300 transfected cells was counted for each mutant of p27.

Recombinant proteins, microinjection and import assays

HeLa cells were microinjected using a Zeiss Axiovert 10 microscope and an Eppendorf microinjector 5242/Micromanipulator 5171 unit. p27 and p27R90G were expressed as his-tagged proteins in *E.coli* as described previously (Steiner *et al.*, 1995) and coupled to Cy3 or Cy2, respectively, using the Fluorolink kit obtained from Amersham. Labelled proteins were injected at a concentration of 1 mg/ml into 50–150 cells per time point. Cells were fixed in 3.5% formaldehyde/phosphate-buffered saline at the indicated time after injection and subcellular localization of the injected protein was analysed by fluorescence microscopy. *In vitro* nuclear import assays were performed according to Huber *et al.* (1998).

Supplementary data

Supplementary data to this paper are available at *The EMBO Journal* Online.

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