Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27^{Kip1}

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The p27^{Kip1} protein associates with G₁-specific cyclin-CDK complexes and inhibits their catalytic activity. p27^{Kip1} is regulated at various levels, including translation, degradation by the ubiquitin/proteasome pathway and non-covalent sequestration. Here, we describe point mutants of p27 deficient in their interaction with either cyclins (p27^{c-}), CDKs (p27^{k-}) or both (p27^{ck-}), and demonstrate that each contact is critical for kinase inhibition and induction of G₁ arrest. Through its intact cyclin contact, p27^{k-} associated with active cyclin E-CDK2 and, unlike wild type p27, p27^{c-} or p27^{ck-}, was efficiently phosphorylated by CDK2 on a conserved C-terminal CDK target site (TPKK). Retrovirally expressed p27^{k-} was rapidly degraded through the proteasome in Rat1 cells, but was stabilized by secondary mutation of the TPKK site to VPKK. In this experimental setting, exogenous wild-type p27 formed inactive ternary complexes with cellular cyclin E-CDK2, was not degraded through the proteasome, and was not further stabilized by the VPKK mutation. p27^{ck-}, which was not recruited to cyclin E-CDK2, also remained stable *in vivo*. Thus, selective degradation of p27^{k-} depended upon association with active cyclin E-CDK2 and subsequent phosphorylation. Altogether, these data show that p27 must be phosphorylated by CDK2 on the TPKK site in order to be degraded by the proteasome. We propose that cellular p27 must also exist transiently in a cyclin-bound non-inhibitory conformation *in vivo*.

Keywords: CDK/CKI/cyclin/p27/proteasome

Introduction

Progression through the various phases of the mitotic division cycle in all eukaryotic cells depends upon the activity of specific cyclin-cyclin-dependent kinase (CDK) complexes. CDK activity is regulated at multiple levels, including association with different cyclins, phosphorylation/dephosphorylation and association with a group of inhibitory proteins collectively called CKIs (reviewed by Morgan, 1995; Sherr and Roberts, 1995). Selective and time-controlled degradation of several of these regulatory proteins by the ubiquitin/proteasome pathway is also emerging as a major and universal mechanism controlling irreversible transitions during the cell cycle (reviewed by

King *et al.*, 1996; Nasmyth, 1996). Ubiquitination of cellular proteins requires the activity of three classes of enzymes: a ubiquitin activating enzyme (or E1), a ubiquitin conjugating enzyme (or E2) and a ubiquitin-protein ligase (or E3). Polyubiquitinated polypeptides are recognized as substrates by the proteasome, a large protein-degrading complex. This processing pathway degrades a large number of specific cellular proteins, and thus participates in many physiological processes (for reviews: Hershko and Ciechanover, 1992; Jentsch, 1992; Ciechanover, 1994; Isaksson *et al.*, 1996; Pahl and Baeuerle, 1996).

Two major phases of the eukaryotic cell cycle, mitosis and the G₁-S transition, are associated with selective protein degradation. During mitosis, chromosome separation at the metaphase-anaphase transition requires degradation of the yeast Pds1p protein (Cohen-Fix *et al.*, 1996) and possibly of additional, yet unidentified proteins. At the end of mitosis, inactivation of CDK1 (Cdc28, CDC2) is brought about by degradation of mitotic cyclins. Ubiquitination of these mitotic targets is dependent upon the activity of a multiprotein E3 complex known as the cyclosome, or anaphase-promoting complex (APC; reviewed by King *et al.*, 1996; Nasmyth, 1996). In both yeast and mammalian cells, degradation of mitotic cyclins by the APC is sustained until late G₁, preventing premature accumulation of mitotic cyclins (Amon *et al.*, 1994; Brandeis and Hunt, 1996). A second complex, different from the APC, directs distinct substrates to the ubiquitin/proteasome pathway in late G₁. In yeast, this complex is required for S-phase entry and includes the products of the CDC34, CDC4, CDC53 and SKP1 genes (Bai *et al.*, 1996; Mathias *et al.*, 1996; Willems *et al.*, 1996; reviewed by Jackson, 1996; King *et al.*, 1996; Nasmyth, 1996). Genetic data imply that the essential substrate of this pathway for the G₁-S transition is SIC1, an inhibitor of S-phase cyclin-CDK complexes (Nugroho and Mendenhall, 1994; Schwob *et al.*, 1994). Other substrates include FAR1 (S.Henchoz, Y.Chi, B.Catarin, I.Herskowitz, R.Deshaies and M.Peter, submitted)—a CKI involved in pheromone-induced arrest (Peter and Herskowitz, 1994)—and G₁ cyclins (CLN2 and 3) (Deshaies *et al.*, 1995; Yaglom *et al.*, 1995). In mammalian cells, both cyclin E and the CKI p27^{Kip1} (p27) are substrates of the ubiquitin/proteasome pathway (Pagano *et al.*, 1995; Clurman *et al.*, 1996; Won and Reed, 1996). Whether the mammalian counterpart of the CDC34 pathway mediates cyclin E or p27 degradation *in vivo* remains unclear, but the human CDC34 homologue Ubc3 can mediate p27 ubiquitination *in vitro* (Pagano *et al.*, 1995).

For yeast CLN2, CLN3, possibly also CLN1, as well as for mammalian cyclin E, ubiquitin-dependent degradation requires site-specific phosphorylation by CDKs (Deshaies *et al.*, 1995; Yaglom *et al.*, 1995; Blondel and Mann, 1996; Clurman *et al.*, 1996; Lanker *et al.*, 1996; Won and

Reed, 1996). Phosphorylated CLN2 is found selectively in a complex with the CDC53 protein, which itself interacts with CDC34 (Willems *et al.*, 1996). Recent evidence suggests that degradation of SIC1 also depends on CDK1-mediated phosphorylation and that this may constitute the essential function of G₁ cyclins for S-phase entry (Schneider *et al.*, 1996; Tyers, 1996; reviewed by King *et al.*, 1996; Nasmyth, 1996). Furthermore, FAR1 degradation also depends upon phosphorylation by CDK1 (S.Henchoz *et al.*, submitted). Whether p27 is also degraded in a phosphorylation-dependent manner has remained unclear.

p27 associates with complexes of cyclins D1–3 with CDK4 or CDK6, and cyclins E or A with CDK2 (Hengst *et al.*, 1994; Kato *et al.*, 1994; Nourse *et al.*, 1994; Polyak *et al.*, 1994a,b; Slingerland *et al.*, 1994; Toyoshima and Hunter, 1994; Poon *et al.*, 1995; Reynisdottir *et al.*, 1995; Sherr and Roberts, 1995; Soos *et al.*, 1996; Vlach *et al.*, 1996). Our own studies of Rat1 cells arrested in G₁ by retrovirally expressed p27 suggested that cyclin E–CDK2 is an essential p27 target *in vivo* (Vlach *et al.*, 1996). Numerous studies suggested a role for p27 in the response of cells to antiproliferative stimuli (Coats *et al.*, 1996; Rivard *et al.*, 1996; reviewed by Sherr and Roberts, 1995). In various—but importantly not all—cellular systems examined, p27 levels decrease following mitogenic stimulation of quiescent cells or, conversely, increase following contact inhibition, mitogen withdrawal or other antiproliferative signals (Kato *et al.*, 1994; Nourse *et al.*, 1994; Halevy *et al.*, 1995; Poon *et al.*, 1995; Coats *et al.*, 1996; Hengst and Reed, 1996; Rivard *et al.*, 1996; Vlach *et al.*, 1996). p27 may also play an important role in the regulation of the ongoing cell cycle, since its levels limit the rate of G₁ progression in Balb/c 3T3 fibroblasts (Coats *et al.*, 1996). Furthermore, the hallmark phenotype of p27-deficient mice is an increase in size, attributable to increased cellular proliferation but without any obvious impairment in terminal differentiation (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). In addition to ubiquitination (Pagano *et al.*, 1995), p27 is regulated at the translational level (Agrawal *et al.*, 1996; Hengst and Reed, 1996) and by non-covalent sequestration, which prevents inhibition of cyclin E–CDK2. The latter can be mediated by D-type cyclins (Polyak *et al.*, 1994a; Poon *et al.*, 1995; Reynisdottir *et al.*, 1995; Sherr and Roberts, 1995; Soos *et al.*, 1996) or, through a distinct and yet unknown intermediate, by c-Myc (Vlach *et al.*, 1996).

At the outset of this work, we sought to understand the role of specific molecular interactions in p27 function and regulation. Our strategy was to design specific mutants of p27 affecting selected protein–protein contacts and to characterize their behaviour both *in vitro* and *in vivo*, by retroviral transduction in cultured cells (Vlach *et al.*, 1996). Our results provide genetic and biochemical evidence that in order to be degraded by the proteasome, p27 must associate with cyclin E–CDK2 complexes in a non-inhibitory mode and be phosphorylated by CDK2 within these complexes on a specific threonine residue. Thus, phosphorylation-dependent degradation of cyclins and CKIs appears to be a universally conserved mechanism. Finally, we also discuss evidence that p27 sequestration induced by c-Myc and phosphorylation-induced degradation are two independent molecular events.

Results

Cyclin- and CDK-specific interaction mutants of p27

The mammalian CKIs p27^{Kip1}, p21^{Cip1/WAF1} and p57^{Kip2} share a conserved domain responsible for binding and inhibiting G₁-specific cyclin–CDK complexes (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994; Goubin and Ducommun, 1995; Lee *et al.*, 1995; Luo *et al.*, 1995; Matsuoka *et al.*, 1995; Nakanishi *et al.*, 1995; Sherr and Roberts, 1995; Fotadar *et al.*, 1996; Lin *et al.*, 1996). Related gene products from other species, such as *Xenopus laevis* p27^{XIC1} (Su *et al.*, 1995), two putative *Caenorhabditis elegans* proteins (acc. No. Z50796) and to a lesser extent *Saccharomyces cerevisiae* SIC1 (Nugroho and Mendenhall, 1994) share sequence homology with this domain (Figure 1A). Based on these observations, we mutagenized murine p27 by double alanine substitutions, replacing arginine 30 and leucine 32 in p27^{c-}, or phenylalanines 62 and 64 in p27^{k-} (Figure 1A). All four residues were mutated in p27^{ck-}. The crystal structure of p27–cyclin A–CDK2 complexes (Russo *et al.*, 1996) confirmed that p27^{c-} and p27^{k-} were altered in cyclin- and CDK-contacting residues, respectively. These residues are also conserved in the recently identified *Drosophila* CKI Dacapo (de Nooij *et al.*, 1996; Lane *et al.*, 1996) (Figure 1A).

The specificity of p27 mutations was addressed by co-incubating GST–p27 fusion proteins expressed in *Escherichia coli* with human cyclin E and CDK2 expressed in Sf9 cells; complexes were pulled down with glutathione beads and the associated cyclin and/or CDK was revealed by immunoblotting. GST–p27^{wt} associated independently with cyclin E or CDK2 (Figure 2A, lane 2). GST–p27^{c-} associated only with CDK2, and GST–p27^{k-} only with cyclin E (lanes 3 and 4). When incubated with cyclin E–CDK2 complexes, GST–p27^{k-} co-precipitated both proteins as efficiently as GST–p27^{wt}, indicating formation of stable ternary complexes (Figure 2B, lanes 2 and 4). In contrast, the presence of cyclin E reduced the interaction between GST–p27^{c-} and CDK2 (lane 3), suggesting that this mutant did not efficiently form ternary complexes. GST–p27^{ck-} failed to co-precipitate cyclin E or CDK2, alone or in combination (Figure 2A and B, lane 5). These experiments were repeated with cyclin A–CDK2 and cyclin D1–CDK4 complexes. Only one notable difference was observed, in that GST–p27^{c-} interacted with these complexes as efficiently as GST–p27^{wt} (Figure 2C and D, compare lanes 2 and 3). However, the interaction pattern of individual cyclin D1 or CDK4 with the various GST–p27 proteins was the same as for cyclin E and CDK2, respectively (Figure 2A and D). Baculovirus-expressed cyclin A did not interact with GST–p27 (wt or mutants) in the absence of CDK2. In summary, p27^{c-} and p27^{k-} were deficient in associating with individual cyclins and CDKs, respectively. However, through their reciprocal intact interaction motifs, these mutants still formed stable ternary complexes with cyclin–CDK pairs (with the exception of p27^{c-}–cyclin E–CDK2). The double mutant p27^{ck-} lost the ability to form stable binary or ternary complexes.

Cyclin- and CDK-contacts are required for efficient kinase inhibition by p27

The *in vitro* kinase assays described below (Figures 3–5) were all performed with purified p27 proteins released

Fig. 1. Alignment of (A) Kip/Cip family proteins and (B) the cyclin-binding motif (or RXL motif) of p107, p130 and E2F proteins (see Discussion). The cyclin and CDK-binding domains determined by the crystal structure of p27-cyclin A-CDK2 complexes (p27/A/K2) are shown at the top. The double alanine substitutions introduced in the murine p27 mutants p27^{c-} and p27^{k-} are shown above the sequences. The proteins are named at the left as follows. (A) mp27, murine p27; hp27, human p27; mp57, murine p57; hp21, human p21; XIC1, *X.laavis* p27^{Xic1}; C.e.1 and C.e.2, predicted *C.elegans* CKIs; DAP, *Drosophila melanogaster* DCAPO; SIC1, *S.cerevisiae* SIC1. (B) RXL motifs of human p107, p130, E2F-1, -2 and -3, as indicated. The coordinate of the first residue shown is indicated before each sequence. Residues that are conserved, or relevant conservative changes are shown in bold within the sequences. The residues of SIC1 shown in lower-case letters indicate that no significant conservation may exist between this domain and the corresponding one of other CKIs. CONS.: the residues conserved in four or more (CONS.I), or three (CONS.II) of the CKIs are indicated in the box; the most highly conserved residues are shown in bold. The conserved residues corresponding to those mutated in p27^{c-} and p27^{k-} are shown in black boxes. See text for references.

from GST-p27 by thrombin cleavage. We assayed the CDK-inhibitory activities of p27 mutants by co-incubating cyclin E-CDK2 or cyclin A-CDK2 with serial dilutions of the p27 proteins and performing histone H1-kinase assays directly in solution. H1-kinase activity was strictly dependent upon the presence of cyclin E or A together with CDK2 in the *Sf9* cell lysates (data not shown). p27^{c-} and p27^{k-} inhibited cyclin E-CDK2 markedly less effectively than p27^{wt}, with half-inhibitory concentrations elevated by about one order of magnitude, whereas p27^{ck-} was non-inhibitory (Figure 3A). p27^{k-} antagonized cyclin A-CDK2 better than cyclin E-CDK2 (Figure 3A and B), although it failed to quantitatively suppress either activity at high concentration (Figure 3C and D, lanes 4). In contrast, p27^{c-} quantitatively inhibited cyclin A-CDK2 at high concentration (Figure 3B and D, lane 3), correlating with the stable association between these proteins (Figure 2C). Upon dilution, however, p27^{c-} reproducibly lost inhibitory activity faster than p27^{k-} (Figure 3B, 10 ng), suggesting that its interaction with the complex was less effective. Results similar to those shown in Figure 3 (C and D) were obtained with kinase assays performed after immunoprecipitation of CDK2 or cyclins in the presence of GST-p27 proteins. Furthermore, the inhibitory pattern of GST-p27 mutants on cyclin D1-CDK4 was comparable with that on cyclin A-CDK2 (data not shown). In conclusion, both cyclin- and CDK-contacts were critical for the function of p27 as a CDK inhibitor.

Site-specific phosphorylation of p27^{k-} by CDK2

In the above experiments, we noted that p27^{k-} was selectively phosphorylated by cyclin E-CDK2 in histone

H1-kinase assays (Figure 3C, lane 4). p27 contains three sites that conform with the minimal Ser/Thr-Pro (S/T-P) consensus CDK phosphorylation site. In murine p27, these are serine 10 (SPSL), serine 178 (SPNA) and threonine 187 (TPKK). Mutation of Thr187 to valine in p27^{k-} (mutant p27^{k-V}) reduced its phosphorylation to background levels (Figure 3C, lane 6). In contrast, mutation of both Ser10 and Ser178 (mutant p27^{k-AA}) had no effect on phosphate incorporation (lane 7). The interaction patterns and H1-kinase-inhibitory activities of p27^{k-V} and p27^{k-AA} were identical to those of p27^{k-} (Figures 2A-D and 3A-D; data not shown). Thus, p27^{k-} was phosphorylated on Thr187 by cyclin E-CDK2.

The phosphorylation of p27 proteins was further investigated by co-incubation with cyclin-CDK2 complexes followed by kinase assays in the absence of histone H1. Neither p27^{wt} nor p27^{c-} were significantly phosphorylated (Figure 4A, lanes 2 and 3). At high p27 concentrations, both p27^{k-} and p27^{ck-} were phosphorylated by cyclin E-CDK2 (Figure 4A, panel i, lanes 4 and 5, and B, lanes 1 and 2). This suggested that the lack of p27^{ck-} phosphorylation in the presence of excess (5 µg) histone H1 (Figure 3C, lane 5) was most likely due to substrate competition, and that the recruitment of p27^{k-} through interaction with cyclin E allowed its phosphorylation under both conditions. Furthermore, when p27 proteins were diluted 10-fold in the H1-free kinase reaction, p27^{ck-} phosphorylation by cyclin E-CDK2 was lost, whereas p27^{k-} phosphorylation was unaltered (Figure 4B, lanes 3 and 4), showing that recruitment became rate-limiting for phosphorylation. The same experiments were repeated with cyclin A-CDK2, with one notable difference: while

Fig. 2. Interaction of p27 mutants with cyclins and CDKs. The point mutations in p27^c, p27^k, p27^{ck} and p27^{k-v} are defined in the text. These mutants as well as p27^{wt} were expressed in *E. coli* as GST-p27 fusion proteins. Human cyclins and CDKs were expressed with recombinant baculoviruses in insect cells. *E. coli* and *Sf9* lysates were mixed at 1:1 ratios (see Materials and methods) and co-incubated. GST proteins were pulled down with glutathione-agarose beads, and the co-precipitated cyclins and CDKs were visualized by immunoblotting. The GST fusion proteins used are indicated at the top of the figure (lanes 1–6). The co-incubated *Sf9*-expressed proteins were: (A) cyclin E or CDK2 alone; (B) cyclin E-CDK2 complexes; (C) cyclin A-CDK2 complexes; (D) cyclin D1 or CDK4 alone, or cyclin D1-CDK4 complexes, as indicated. In (C), the anti-cyclin A antibody cross-reacted with GST and GST-p27 proteins, as indicated.

p27^{ck} was phosphorylated by this complex as efficiently as by cyclin E-CDK2, p27^k was a poor substrate (Figure 4A, lanes 4 and 5, and B, lanes 5 and 6). As with cyclin E-CDK2, phosphorylation of p27^{ck} by cyclin A-CDK2 was lost upon dilution (Figure 4B, lanes 6 and 8), or in the presence of histone H1 (Figure 3D, lane 5). As control, none of the p27 proteins were phosphorylated in *Sf9* lysates lacking cyclin-CDK complexes (Figure 4B, lane 9; data not shown). In summary, the non-interacting mutant p27^{ck} was phosphorylated by cyclin E-CDK2 or cyclin A-CDK2 *in vitro* in a concentration-dependent manner, analogous to histone H1. On the other hand, recruitment of p27^k through the cyclins suppressed its phosphorylation by cyclin A-CDK2, but favoured its phosphorylation by cyclin E-CDK2 at lower concentrations. Comparison of p27^k with the phosphosite mutants p27^{k-v} and p27^{k-AA} showed that p27^k phosphorylation occurred on Thr187 (Figure 4A, lanes 4, 6 and 7). Finally, cyclin D1-CDK4 phosphorylated all p27 proteins (wt, c-, k- or ck-) with

Fig. 3. Inhibitory effect of p27 mutants on the histone H1-kinase activities of cyclin-CDK2 complexes. The indicated amounts of purified p27 proteins (released from GST-p27 by thrombin cleavage) were co-incubated with (A) cyclin E-CDK2 or (B) cyclin A-CDK2 complexes prior to kinase reactions. 1000 ng corresponded to a p27 concentration of 900 nM in the kinase assay. ³²P incorporation in histone H1 for each reaction was quantified with a phosphorimager following gel electrophoresis, and normalized to the activity of each cyclin-CDK2 complex alone (100%). The curves for the phosphosite mutant p27^{k-AA} (not shown) were superimposable on those of p27^k and p27^{k-v}. The values given are averages from three independent experiments which yielded very close results. (C) and (D) show representative autoradiograms for kinase assays performed with 1000 ng of the indicated p27 proteins, and correspond to the values given in (A) and (B), respectively. The bands corresponding to phosphorylated histone H1 and p27 proteins are indicated.

an equal, albeit low efficiency, and on a site distinct from Thr187 (data not shown).

p27^k associates with active cyclin-CDK2 complexes

Altogether, the above data implied that p27^k should associate with kinase-active cyclin E-CDK2. To address this point directly, we immunoprecipitated ternary complexes with anti-p27 antibodies, and performed histone H1-kinase assays. Complexes formed with p27^{wt} were devoid of any kinase activity (Figure 5, lane 2). In contrast, p27^k co-precipitated H1-kinase activity (lane 4). This activity was significantly above background levels (lane 1), was eliminated by peptide-blocking of the anti-p27 antibody (lane 8), and was dependent upon cyclin E and CDK2 in *Sf9* lysates (data not shown). Furthermore, this activity was not due to release of the kinase from p27^k during incubation, since it was fully resistant to pre-incubation and washes in kinase-reaction buffer (data not shown). p27^k-associated activity was not dependent upon the presence of the phosphoacceptor site Thr187, since p27^{k-v} also bound active complexes (Figure 5, lane 6) (the same was true for the Ser 10/178 mutant p27^{k-AA}: lane 7). Neither p27^c nor p27^{ck} associated with active CDK2 (Figure 5, lanes 3 and 5). Thus, p27^k associated with active CDK2 through its direct contact with cyclin E.

In apparent conflict with this finding, we show in Figure 3A that p27^k had a residual inhibitory activity on cyclin E-CDK2. However, both sets of data were highly reproducible, either with purified p27 or with GST-p27 proteins. This implies that p27^k-associated cyclin E-CDK2 is

Fig. 4. Phosphorylation of p27 proteins in the absence of histone H1. (A) The indicated cyclin-CDK2 complexes were incubated alone (lanes 1) or in the presence of 1000 ng (900 nM) of the indicated purified p27 proteins (lanes 2–7), and kinase assays were performed without histone H1. Phosphorylated p27 proteins are shown. (B) As above, but 1000 or 100 ng of p27^{k-} or p27^{ck-} were incubated with cyclin-CDK2 complexes, as indicated.

Fig. 5. Association of p27^{k-} with active cyclin E-CDK2. The indicated p27 proteins were incubated with cyclin E-CDK2 complexes, followed by immunoprecipitation with the anti-p27 antibody C-19. Histone H1-kinase assays were performed on the immunoprecipitates. Phosphorylated histone H1 is shown. Lane 8, C-19 was blocked with its cognate peptide prior to immunoprecipitation.

only partially active in phosphorylating histone H1, in comparison with the free kinase complex. This may be due to substrate competition between p27^{k-} and H1, residual inhibitory activity of p27^{k-}, or both.

Cyclin A-CDK2 was unable to phosphorylate histone H1 when associated with p27^{k-} (data not shown). This correlated with the inability of cyclin A-CDK2 to phosphorylate p27^{k-} (Figures 3D and 4A) and with its more efficient inhibition by p27^{k-} in solution (Figure 3B). Altogether, these observations implied that the effect of the p27^{k-} mutation on CDK2 inhibition was less severe if cyclin A was present in the complex.

Cyclin- and CDK-contacts are critical for p27-induced G₁ arrest

To assess the biological function of p27 mutants, we expressed these proteins in Rat1 cells from a retroviral

Fig. 6. Effects of p27 mutants on cyclin-CDK2 activity and S-phase entry in infected cells. Cells were infected with a control pBabePuro retrovirus (lane 1) or with retroviruses expressing p27^{wt}, p27^{c-}, p27^{k-}, p27^{ck-} or p27^{k-V} (lanes 2–6), as indicated. Puromycin-resistant cells were harvested 48 h post-infection, as previously described (Vlach *et al.*, 1996). Immunoprecipitations (IP) were performed from cell lysates (15 µg) with antibodies against cyclin E or cyclin A, as indicated to the left. Histone H1-kinase assays were performed on the immunoprecipitates. (B) Percentage of cells in S-phase 48 h post-infection. S-phase cells were visualized by immunocytochemical detection of incorporated BrdU.

vector. p27^{wt} directly inhibited cyclin E-CDK2 and caused G₁ arrest. Cyclin A mRNA and protein levels were suppressed in these cells, and thus also cyclin A-CDK2 activity (Vlach *et al.*, 1996). In contrast, viruses expressing p27^{c-}, p27^{k-} or p27^{ck-} induced no significant suppression of cellular cyclin E- and A-associated kinase activities (Figure 6A), cell cycle progression (Figure 6B) or long-term proliferation (data not shown).

p27^{k-} is degraded at an enhanced rate in vivo

The expression of p27 mutants was assessed by immunoblotting (Figure 7A). We previously showed that retroviral p27^{wt} was expressed at physiological levels (comparable with those of endogenous p27 in contact-inhibited cells; Vlach *et al.*, 1996). In comparison with p27^{wt} (lane 2), p27^{c-} levels were unaltered and p27^{ck-} levels were slightly enhanced (lanes 3 and 5). In contrast, p27^{k-} accumulated in cells at low levels (lane 4). CDK2 levels were the same in all cell populations (Figure 7A). Decreased accumulation of p27^{k-} was unlikely to result from differences in translational control (Hengst and Reed, 1996),

Fig. 7. Levels of recombinant p27 proteins in retrovirally infected Rat1 cells. (A) Cells were infected as explained in Figure 6. p27 levels in puromycin-selected infected cells were assessed by immunoblot analysis with the C-19 antibody 2 days post-infection (top panel). Probing with the Neo Markers monoclonal antibody yielded an identical pattern. The same blot was reprobed with an antibody against CDK2 (bottom panel). The visualized proteins are indicated to the left. (B) Same as above, comparing p27^{wt} with the Val187 mutant p27^{V187}.

since only the open reading frame of p27 was included in our constructs. Furthermore, retroviral mRNA levels in infected cells were the same for all mutants (data not shown). Pulse-chase analysis of ³⁵S-labelled, retrovirally infected cells showed that p27^{k-} was degraded at an enhanced rate in comparison with p27^{wt} (Figure 8A, panels i and ii).

Degradation of p27^{k-} requires the Thr187 phosphoacceptor site

Together with our *in vitro* data, the above observations suggested that phosphorylation of p27^{k-} by cyclin E-CDK2 accounted for its instability *in vivo*. In this case, mutation of Thr187 to valine (as in p27^{k-v}) should have restabilized the protein. This point was demonstrated. First, p27^{k-v} accumulated at levels higher than those of p27^{k-} and comparable with those of p27^{wt} (Figure 7A, lanes 2, 4 and 6). Yet, like p27^{k-}, p27^{k-v} failed to suppress cellular cyclin A- and E-associated kinase activities or cell cycle progression (Figure 6). In contrast to Thr187, mutation of alanines 10, 178 or both in p27^{k-} had no significant effects on the accumulation of the protein (data not shown). Second, p27^{k-v} remained stable throughout the time course of our ³⁵S-pulse-chase experiments (Figure 8A, panel iii).

To attempt a direct visualization of Thr187 phosphorylation in p27^{k-}, we metabolically labelled infected cells with [³²P]orthophosphate. However, no labelling of p27^{k-} could be detected (Figure 8B, lane 2), most likely owing to the high degradation rate of this protein once phosphorylated. Furthermore, p27^{wt}, p27^{c-}, p27^{ck-} and the phosphosite mutant p27^{k-v} incorporated ³²P at equal levels (Figure 8B, lanes 3–5; data not shown). Thus, p27 is phosphorylated *in vivo* on additional site(s) distinct from Thr187, and these phosphorylation events are independent of cyclin- and CDK-contacts. These circumstances precluded direct visualization of Thr187 phosphorylation in p27^{k-}.

Fig. 8. Metabolic labelling of recombinant p27 proteins in retrovirally infected cells. (A) Half-life of (i) p27^{k-}, (ii) p27^{wt} and (iii) p27^{k-v} *in vivo*. Infected cells were labelled for 4 h with [³⁵S]cysteine and [³⁵S]methionine. Radiolabelled cells were washed, incubated in fresh medium and harvested at the indicated time points. p27 proteins were immunoprecipitated with C-19 and resolved by gel electrophoresis. (B) Phosphorylation of p27 proteins *in vivo*. Infected cells expressing the indicated proteins were labelled for 4 h with [³²P]orthophosphate and harvested. Labelled p27 proteins were visualized as in (A).

Fig. 9. p27^{k-}-associated histone H1-kinase activity in infected cells. Cells were infected as explained in Figure 6. Immunoprecipitations were performed on cell lysates with the C-19 antibody against p27. Histone H1-kinase assays were performed on the immunoprecipitates. (B) Equal amounts of p27^{k-} were recovered from infected cells in several parallel immunoprecipitations. H1-kinase reactions were performed in the presence of the CDK-inhibitory drugs Flavopiridol or Roscovitine at the concentrations indicated (5 and 0.5 μM).

p27^{k-} associates with active CDK2 *in vivo*

Since p27^{k-} associated with active cyclin E-CDK2 *in vitro* (Figure 5), we investigated whether this could happen *in vivo*. To this end, retrovirally expressed p27 proteins were immunoprecipitated from cells, followed by H1-kinase assays. As expected from the *in vitro* results, p27^{wt}, p27^{c-} and p27^{ck-} associated with no significant kinase activity (Figure 9A, lanes 2, 3 and 5). In contrast,

Fig. 10. p27^{k-} is degraded through the proteasome *in vivo*. (A) Cells infected with the indicated retroviruses were treated with DMSO (-) or with the proteasome inhibitor LLNL (final concentration 50 μ M) (+) for 4.5 h prior to harvesting and immunoblot analysis of p27 levels (as in Figure 7). (B) As above, but comparing the inhibitors LLNL, LLM, E-64 and NCO-700 (NCO), as indicated. Cells were treated for 6 h with LLNL, LLM and E-64 at a final concentration of 50 μ M, and with NCO-700 at a concentration of 100 μ M. Immunoblots were exposed for (i) 15 s and (ii) 4 min.

p27^{k-} co-precipitated active H1-kinase (lane 4). This was observed with two anti-p27 antibodies (C-19 and Neo Markers), and no kinase activity was recovered when the C-19 antibody was blocked with its cognate peptide (data not shown).

Cellular cyclin E and CDK2 associated with exogenous p27^{wt} could be detected by immunoprecipitation followed by immunoblot analysis (Vlach *et al.*, 1996). However, decreased levels of p27^{k-} precluded detection of these interactions (data not shown). To identify the cellular kinase associated with p27^{k-}, we treated kinase reactions with the CDK-inhibitory drugs Flavopiridol and Roscovitine. Both drugs eliminated kinase activity in p27^{k-} immunoprecipitates (Figure 9B). At the concentrations used, Flavopiridol inhibits both CDK2 and CDK4, whereas Roscovitine inhibits only CDK2 (reviewed by Meijer, 1996). Together with our *in vitro* studies, these data imply that p27^{k-} associates in cells with active CDK2 complexes, most likely through its recruitment by cyclin E.

p27^{k-} degradation is mediated by the proteasome

p27 is a known substrate of the ubiquitin/proteasome pathway (Pagano *et al.*, 1995). To test whether this pathway was responsible for degradation of p27^{k-}, we treated infected cells with the proteasome inhibitor LLNL (Rock *et al.*, 1994). This resulted in increased accumulation of p27^{k-}, but not of p27^{wt}, p27^{ck-} or p27^{c-} (Figure 10A and data not shown). Since LLNL also inhibits calpain, we tested the calpain-specific inhibitors LLM, E-64 and NCO-700 (see Materials and methods). Only LLNL had a significant stabilizing effect on p27^{k-} (Figure 10B, panel i, lanes 6–10). Longer exposure of the immunoblot showed

that endogenous p27 in control cells was specifically stabilized by LLNL (panel ii, lanes 1–5), in a manner analogous to p27^{k-} and consistent with previous data (Pagano *et al.*, 1995). That endogenous p27^{wt} did not reach total levels comparable with those of p27^{k-} in the presence of LLNL was expected, since the rate of synthesis of endogenous p27 was very low in growing cells, as demonstrated by our *in vivo* ³⁵S-labelling experiments (Figure 8A, panel i: compare 'vector' with p27^{k-}, in the absence of chase). Altogether, our data show that the decreased expression of p27^{k-} is due to enhanced degradation by the proteasome.

Retrovirally expressed wild-type p27 remains stable in G₁-arrested cells

Like retrovirally expressed p27^{k-}, endogenous p27^{wt} in growing cells turned over through the proteasome (Figure 10B) (Pagano *et al.*, 1995). However, retrovirally expressed p27^{wt} was not degraded at a high rate, as shown by immunoblotting (Figure 7A, lane 2), pulse-chase experiments (Figure 8A, panel ii) and by the fact that LLNL did not increase its levels (Figure 10A). As already mentioned, exogenous p27^{wt} associated with cyclin E-CDK2 in an inactive form (Figure 9A) (Vlach *et al.*, 1996) and, under these circumstances, did not become phosphorylated on Thr187 (Figure 4). Consistent with these observations, mutation of Thr187 in the context of wild-type p27 (mutant p27^{V187}) did not stabilize the protein *in vivo* (Figure 7B), and did not alter its CDK- and growth-inhibitory activities (data not shown).

In summary, the outcome of ectopic p27^{wt} expression was the formation of inactive complexes with cyclin E-CDK2, with ensuing lack of phosphorylation on Thr187 and lack of targeting to the proteasome. In contrast, through its interaction with cyclin E, the CDK-contact mutant p27^{k-} was recruited to active CDK2, phosphorylated on Thr187 and degraded by the proteasome. Altogether, our data imply that p27 degradation in cells requires its transitory association with active cyclin E-CDK2 and phosphorylation on Thr187, and suggest that this process might be regulated by additional signals.

Discussion

Cyclin- and CDK-interaction mutants of p27^{Kip1}: implications for cyclin-CDK2 function and specificity

We have constructed two mutants of murine p27^{Kip1} specifically deficient in interacting with cyclins (p27^{c-}) and CDKs (p27^{k-}), and the corresponding double mutant (p27^{ck-}) defective in both interactions. Using these mutants, we demonstrated that contacts with both cyclin and CDK subunits are required for the optimal function of p27 as a kinase inhibitor. Two residues were mutated to alanines in each mutant: arginine 30/leucine 32 in p27^{c-} and phenylalanines 62/64 in p27^{k-} (Figure 1A). These residues lie within the previously mapped cyclin-CDK-binding domains of mammalian Kip/Cip family proteins, and are part of conserved sequence motifs in all CKIs of this family, including p21^{Cip1}, p27^{Kip1}, p57^{Kip2}, *Xenopus* p27^{Xic1}, two putative *C.elegans* gene products, as well as the *Drosophila* CKI *Dacapo* (Figure 1A, see references in first paragraph of Results). The crystal structure of

p27–cyclin A–CDK2 ternary complexes showed that the targeted residues contact the cyclin and CDK subunits, as predicted (Russo *et al.*, 1996). We propose in Figure 1A a possible alignment of the yeast CKI SIC1 with the Kip/Cip family. It will be of interest to address whether mutations equivalent to those in p27^{c-} or p27^{k-} affect interaction of SIC1 with its cyclin and CDK partners in yeast.

The cyclin-binding motif (or RXL motif) mutated in p27^{c-} is found in proteins other than CKIs, including the retinoblastoma family members p107 and p130, as well as E2F-1, -2 and -3 (Figure 1B) (Zhu *et al.*, 1995; Adams *et al.*, 1996). Several observations suggest that these different RXL motifs indeed bind a common determinant on cyclins. Peptides spanning the RXL motif from representative members of all groups shown in Figure 1 can compete with recombinant GST–p21 or GST–E2F-1 for interaction with cyclins. Mutations of the conserved Arg or Leu to Ala in E2F-1-derived peptides, which correspond to the two substitutions introduced here in p27^{c-}, eliminated their competing activity (Adams *et al.*, 1996). Besides the RXL motif, p107, p130 or E2F proteins lack any obvious CDK-contact motif and can act as targets or tethers for active CDK2. For example, binding of cyclin A–CDK2 to E2F-1/DP-1 dimers results in their phosphorylation and suppression of DNA-binding activity, a mechanism thought to play an important role in downregulation of E2F function during S-phase (Dynlacht *et al.*, 1994; Krek *et al.*, 1994).

Consistent with the notion that the RXL motif can target substrates to active cyclin–CDK complexes, the CDK-contact mutant p27^{k-} (in which the RXL motif was intact; Figure 1A) bound to active cyclin E–CDK2 and was efficiently phosphorylated on Thr187. Further mutation of the RXL motif, such as in the double mutant p27^{ck-}, led to a loss of phosphorylation at limiting substrate concentrations. At high concentrations, p27^{ck-} was phosphorylated by both cyclin E–CDK2 and cyclin A–CDK2, analogous to histone H1. Interestingly, p27^{k-} also associated with cyclin A–CDK2, but was not significantly phosphorylated by this complex. Thus, recruitment of p27^{k-} through the cyclin was inhibitory for Thr187 phosphorylation in the case of cyclin A, but stimulatory in the case of cyclin E. This stimulatory effect became evident at low p27^{k-} concentrations or in the presence of excess histone H1, indicating that interaction with cyclin E became rate-limiting for phosphorylation under these conditions. In our experiments, neither cyclin E–CDK2 nor cyclin A–CDK2 phosphorylated p27^{wt}, correlating with the formation of inactive ternary complexes. Cyclin D1–CDK4 phosphorylated p27^{wt}, p27^{c-}, p27^{k-}, p27^{ck-} or the Ala187 derivative of p27^{k-} (p27^{k-V}) to identical, albeit very low levels, which may bear no physiological relevance.

While our work was in revision, Sheaff *et al.* (1997) reported that wild-type p27 (p27^{wt}) could be phosphorylated by cyclin E–CDK2 *in vitro* on Thr187. Analysis of phosphorylation kinetics and of the effects of ATP concentration led these authors to propose that p27 bound to cyclin E–CDK2 in two conformations: first in a ‘loose’ state, under which CDK2 phosphorylated p27, and secondly in a ‘tight’ state, under which the kinase was inhibited. The loose state may be analogous to that adopted

by our mutant p27^{k-}, which does not undergo the transition to the inhibitory conformation. The tight state most likely is equivalent to the described structure of p27–cyclin A–CDK2 complexes, in which ATP binding to CDK2 is precluded (Russo *et al.*, 1996). The residues mutated in p27^{k-} (Figure 1A) play an important role in this structure. These observations altogether imply that p27 must transiently exist in a cyclin E-bound non-inhibitory conformation in order to become an effective CDK2 substrate.

The cyclin-contact mutant p27^{c-} also formed stable ternary complexes with cyclin A–CDK2 and cyclin D1–CDK4, in this case through its intact CDK-contact domain. In contrast, the presence of cyclin E reduced interaction of p27^{c-} with CDK2. This suggests either that CDK2 adopts slightly different conformations when bound to cyclin A or E, or that cyclin A can sterically hinder interactions of CDK2 with p27^{c-}. Thus, interaction of CDK2 with other proteins (exemplified here by p27^{c-}) may be differentially affected by cyclin E or A.

Recruitment of p27 to active cyclin E–CDK2 complexes triggers its degradation *in vivo*

When expressed in Rat1 cells with a retroviral vector, p27^{wt} inactivated cyclin E–CDK2 and arrested cells in G₁ (Vlach *et al.*, 1996). All of the p27 mutants described here had lost these properties. While p27^{c-} and p27^{ck-} were expressed *in vivo* at levels equal to, or slightly higher than those of p27^{wt}, the CDK-contact mutant p27^{k-} accumulated to much lower levels *in vivo*. ³⁵S-pulse-chase studies showed that this was due to enhanced degradation of p27^{k-}. Treatment of cells with the proteasome inhibitor LLNL elevated p27^{k-} levels, but had no effect on the other recombinant p27 proteins, showing that p27^{k-} was degraded by the proteasome, as previously shown for cellular p27 (Pagano *et al.*, 1995).

From our *in vitro* data, we predicted that association of p27^{k-} with active cyclin E–CDK2 in cells would lead to phosphorylation on Thr187, and that this effect would trigger the enhanced turnover of the protein. We were able to prove the validity of this hypothesis. First, consistent with our *in vitro* data, we observed that p27^{k-} recovered from cells was associated with catalytically active CDK2. No significant kinase activity was recovered with p27^{wt}, p27^{c-} or p27^{ck-} from cells, even though those proteins were expressed at higher levels. Second, mutation of threonine 187 to valine in p27^{k-} (mutant p27^{k-V}) led to restabilization of the protein, without preventing its stable association with active cyclin–CDK2 complexes. Thus, although we could not biochemically detect phosphorylation of Thr187 *in vivo* (see Results), our data demonstrated that this phosphosite was required for degradation. Third, degradation of p27^{k-} was strictly dependent upon interaction with cyclin E, since the double mutant p27^{ck-} which did not bind cyclin E–CDK2 complexes was not degraded by the proteasome. In addition, we showed *in vitro* that although both cyclin E and cyclin A recruited p27^{k-} onto CDK2, only cyclin E–CDK2 efficiently phosphorylated p27^{k-}. Cyclin D1–CDK4 phosphorylated p27^{wt} and all the mutants very inefficiently and with no discrimination. This implies that cyclin E, rather than cyclin A or D1, was responsible for the turnover of p27^{k-} *in vivo*.

Our results with p27^{k-} imply that cellular p27, in order to be degraded *in vivo*, must be phosphorylated by CDK2

on Thr187. This model predicts two possible, alternative outcomes upon expression of recombinant p27^{wt} in cells. In the first, p27^{wt} would form inactive ternary complexes with cyclin E-CDK2, would not become phosphorylated on Thr187 and as a consequence would not be targeted to degradation by the proteasome. Consistent with this scenario, retrovirally expressed p27^{wt} associated with inactive cyclin E-CDK2, was stable in our pulse-chase analysis, and was not further stabilized by proteasome inhibitors or by mutation of Thr187. In this system, co-expression of cyclin E with p27^{wt} at moderate levels was not sufficient to bypass CDK2 inhibition, destabilize p27^{wt} or overcome G₁ arrest (Vlach *et al.*, 1996). In the second outcome, ectopically expressed p27^{wt} would be phosphorylated by cyclin E-CDK2 on Thr187 and would be rapidly degraded. This was observed in the parallel work of Sheaff *et al.* (1997), who showed that p27^{wt} expressed by transient transfection was eliminated from cells by co-expression of cyclin E. By generating a high and sudden burst of cyclin E activity, these authors were able to demonstrate that phosphorylation of p27^{wt} on Thr187 preceded its elimination *in vivo*. As expected, cyclin E suppressed p27-induced G₁ arrest in those experiments. Thus, the two complementary approaches used by ourselves and by Sheaff *et al.* (1997) verify the two major predictions of the proposed model, and establish that phosphorylation of p27 on its carboxy-terminal TPKK site is a prerequisite for its degradation through the proteasome. It is particularly noteworthy that the TPKK site lies within a conserved homology domain found at the carboxy-termini of p27 and p57 (Lee *et al.*, 1995; Matsuoka *et al.*, 1995), suggesting that the two proteins may be subject to similar control mechanisms.

The half-life of p27 increases upon contact-inhibition in HS68 cells and, conversely, decreases upon S-phase arrest in HeLa cells (Hengst *et al.*, 1994). *In vitro* studies showed that proliferating cell extracts ubiquitinated p27 at higher rates than quiescent extracts (Pagano *et al.*, 1995), and that S-phase extracts degraded p27 whereas mid-G₁ extracts did not (Brandeis and Hunt, 1996). In yeast, G₁-specific CLN-CDK1 activity is required for ubiquitination and degradation of SIC1, most likely through direct SIC1 phosphorylation (Schneider *et al.*, 1996; Tyers, 1996). SIC1 inhibits S-phase-specific CLB-CDK1 complexes. Thus, through the degradation of SIC1, G₁-cyclin-CDK complexes allow activation of S-phase complexes (reviewed by King *et al.*, 1996; Nasmyth, 1996). Our data suggest that degradation of p27 induced by cyclin E-CDK2 may have in part a similar role in preventing inhibition of cyclin A-CDK2 by p27 during S-phase.

Similarly to p27, cyclin E is degraded by the ubiquitin/proteasome pathway following phosphorylation by CDK2 (Clurman *et al.*, 1996; Won and Reed, 1996). A cyclin E mutant deficient in interaction with CDK2 was not phosphorylated, but remained free and was degraded (Clurman *et al.*, 1996). In contrast, our p27^{ck-} mutant, which did not bind cyclin E-CDK2 complexes, was not targeted to degradation. This suggests that the primary effect of phosphorylation is different for cyclin E and p27: to induce release from CDK2 in the case of cyclin E, and to generate a recognition site for components of the degradatory machinery in the case of p27. Alternat-

ively, both mechanisms may be involved in p27 degradation. In yeast, G₁ cyclins and both identified CKIs, SIC1 and FAR1, are targeted to the ubiquitin/proteasome pathway in a phosphorylation-dependent manner (see Introduction). Thus, phosphorylation-dependent degradation of G₁-specific cyclins and CKIs is a fundamentally conserved process.

One question arising here concerns the relationship between the different mechanisms that regulate p27 function in cells. In particular, p27 can be sequestered away from cyclin E-CDK2 complexes, either by D-type cyclins (Polyak *et al.*, 1994a; Poon *et al.*, 1995; Reynisdottir *et al.*, 1995; Sherr and Roberts, 1995; Soos *et al.*, 1996) or by an as yet uncharacterized Myc-induced activity (Vlach *et al.*, 1996). Our own observations suggest that Myc-induced sequestration may be mechanistically unlinked from phosphorylation-induced degradation. First, Myc overrode cell cycle arrest by p27 without decreasing p27 levels or half-life in cells. Secondly, Myc could overcome G₁-arrest induced by the phosphosite mutant p27^{V187} (Vlach *et al.*, 1996 and unpublished data). Thus, rescue of p27-induced G₁ arrest by Myc does not require Thr187 phosphorylation.

Recent immunohistochemical studies showed that a decrease in p27 levels is an indicator of poor prognosis in human breast and colon carcinomas (Catzavelos *et al.*, 1997; Fredersdorf *et al.*, 1997; Loda *et al.*, 1997; Porter *et al.*, 1997). It was further suggested that this decrease may be due to enhanced p27 degradation in high-grade tumor cells (Loda *et al.*, 1997). Thus, although p27 itself is not the product of a tumor suppressor gene (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996; reviewed by Harper and Elledge, 1996), deregulation of p27 degradation and/or sequestration (induced by Myc or D-type cyclins) may play a major role in tumorigenesis. The precise interplay between these p27-regulatory pathways remains to be elucidated, and will require identification of the cellular proteins that associate with p27 in response to either activation of Myc or phosphorylation by CDK2.

Materials and methods

Cells, recombinant retroviruses and infections

Mutant p27 cDNAs (see text) were constructed using PCR and were subcloned in the retroviral vector pBabePuro. Retroviruses were generated and used to infect Rat1 cells following a previously described protocol (Vlach *et al.*, 1996).

Chemical inhibitors

Flavopiridol (NSC 649890) was obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Roscovitine was a gift from Dr Laurent Meijer (Roscoff). LLNL (*N*-acetyl-Leu-Leu-norleucinal) was purchased from Sigma (A-6185). LLM (*N*-acetyl-Leu-Leu-methioninal), E-64 and NCO-700 were purchased from Calbiochem (Nos 208721, 324890 and 479919, respectively).

Recombinant proteins, biochemical studies and antibodies

Mutant p27 cDNAs were subcloned in the vector pGEX-2T and used to express GST fusion proteins in *E. coli* by standard methods (Pharmacia Biotech protocols). After thrombin cleavage, the released p27 proteins were estimated to be over 90% pure. Non-cleaved GST-p27 represented <0.5% of the purified protein. Baculoviruses expressing cyclins and CDKs were used to infect Sf9 cells. Sf9 cells were lysed in the appropriate lysis buffers for CDK2 (Vlach *et al.*, 1996) or CDK4 (Matsushime *et al.*, 1994), at a final total protein concentration of 3 mg/ml. Inhibition of

baculovirus-derived cyclin-CDK complexes by GST-p27 was described previously (Toyoshima and Hunter, 1994). Lysis of retrovirally infected Rat1 cells 2 days post-infection and subsequent biochemical experiments, including immunoprecipitations, immunoblotting and kinase assays, were as previously described (Vlach *et al.*, 1996). The following antibodies were used: against p27, C-19 (Santa Cruz sc-528) or DCS72 (Neo Markers); CDK2, M2 (sc-163); rodent cyclin E, M20 (Santa Cruz sc-481); human cyclin E, C19 (sc-198); cyclin D1, 72-13G (sc-450); CDK4, C-22 (sc-260). For metabolic labelling, infected cells were labelled for 4 h with [³⁵S]methionine/[³⁵S]cysteine (0.11 mCi/ml) or with [³²P]orthophosphate (0.5 mCi/ml).

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