The EMBO Journal Vol. 21 No. 13 pp. 3390–3401, 2002

A growth factor-dependent nuclear kinase phosphorylates p27^{Kip1} and regulates cell cycle progression

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The cyclin-dependent kinase inhibitor, p27^{Kip1}, which regulates cell cycle progression, is controlled by its subcellular localization and subsequent degradation. p27^{Kip1} is phosphorylated on serine 10 (S10) and threonine 187 (T187). Although the role of T187 and its phosphorylation by Cdks is well-known, the kinase that phosphorylates S10 and its effect on cell proliferation has not been defined. Here, we identify the kinase responsible for S10 phosphorylation as human kinase interacting stathmin (hKIS) and show that it regulates cell cycle progression. hKIS is a nuclear protein that binds the C-terminal domain of p27^{Kip1} and phosphorylates it on S10 in *vitro* and *in vivo*, promoting its nuclear export to the cytoplasm. hKIS is activated by mitogens during G_0/G_1, and expression of hKIS overcomes growth arrest induced by p27^{Kip1}. Depletion of KIS using small interfering RNA (siRNA) inhibits S10 phosphorylation and enhances growth arrest. p27$^{-/-}$ cells treated with KIS siRNA grow and progress to S/G_2 similar to control treated cells, implicating p27^{Kip1} as the critical target for KIS. Through phosphorylation of p27^{Kip1} on S10, hKIS regulates cell cycle progression in response to mitogens.

Keywords: cell cycle/hKIS/p27^{Kip1}/phosphorylation/serine

Introduction

The protein p27^{Kip1} is an important regulator of the mammalian cell cycle (Sherr and Roberts, 1999). An increase in p27^{Kip1} causes proliferating cells to exit from the cell cycle, while a decrease in p27^{Kip1} is required for quiescent cells to resume cell division (Loda *et al*., 1997; Porter *et al*., 1997). Low levels of p27^{Kip1} are associated with excessive cell proliferation in pathological conditions such as inflammation and cancers (Fero *et al*., 1998; Ophascharoensuk *et al*., 1998). High levels of p27^{Kip1} are observed in conditions of diminished cell proliferation such as in the late stages of arterial wound repair in atherosclerosis (Tanner *et al*., 1998).

p27^{Kip1} is regulated by transcriptional (Servant *et al*., 2000), translational (Agrawal *et al*., 1996; Hengst and Reed, 1996; Millard *et al*., 1997) and proteolytic mechanisms. A major mechanism in the regulation of p27^{Kip1} abundance is proteolysis by the ubiquitin–proteasome pathway (Pagano *et al*., 1995). Phosphorylation of p27^{Kip1} on threonine 187 (T187) by Cdk2 creates a binding site for a Skp2-containing E3 ubiquitin-protein ligase, SCF (Feldman *et al*., 1997; Skowyrs *et al*., 1997), and ubiquitylation of p27^{Kip1} by SCF results in degradation of p27^{Kip1} by the proteasome (Carrano *et al*., 1999; Sutterluty *et al*., 1999; Tsvetkov *et al*, 1999). This pathway is operational in the S and G_2 phases of the cell cycle, after Cdk2 is activated by cyclins E and A. A second proteolytic pathway for controlling p27^{Kip1} is activated by mitogens and degrades p27^{Kip1} during G_0/G_1 (Malek *et al*., 2001). Inactivation of p27^{Kip1} also occurs by sequestration into cyclin D–Cdk complexes (Sherr and Roberts, 1999).

Serine 10 (S10) is another phosphorylation site on p27^{Kip1} (Ishida *et al*., 2000). Phosphorylation of S10 signals the nuclear export of p27^{Kip1} to the cytoplasm upon cell cycle re-entry (Rodier *et al*., 2001), and it is generally believed that the S10 phosphorylation pathway plays a role in p27^{Kip1} degradation. Despite these observations, the mechanisms regulating S10 phosphorylation are poorly understood. Specifically, the protein that phosphorylates p27^{Kip1} on S10 and its role in the regulation of cell cycle progression have not been defined. Here, we identify the serine-threonine kinase hKIS (human kinase interacting stathmin) as the major kinase that phosphorylates p27^{Kip1} on S10. We demonstrate that phosphorylation of p27^{Kip1} on S10 by hKIS is activated by mitogens in G_0/G_1 cells and that this modification of S10 facilitates nuclear export of p27^{Kip1} to the cytoplasm. In addition, we show that the physiological significance of S10 phosphorylation by hKIS is that it regulates cell cycle progression.

Results

Identification of a serine/threonine kinase, hKIS, which interacts with the C-terminal of p27^{Kip1}

We hypothesized that the C-terminal of p27^{Kip1} would be important in p27^{Kip1} protein–protein interactions, and hence we employed a yeast two-hybrid screen using a human B-cell library (Durfee *et al*., 1993). The yeast two-hybrid screen yielded several cDNAs that interacted with the p27^{Kip1} C-terminal, as well as with full-length p27^{Kip1}, but not the N-terminal region of p27^{Kip1}, p57^{Kip2} or p21^{Cip1} (Figure 1A). One clone, KIS(C21), encoded a 49 kDa protein that is 98% homologous to a rat serine/threonine protein kinase, KIS (DDBJ/EMBL/GenBank accession No. X98374). We determined that this clone was the human homologue of rat KIS, whose function was unknown (Maucuer *et al*., 1995). hKIS has an N-terminal serine/threonine kinase consensus region and a C-terminal region with 42% sequence identity to hU2AF65, a 65 kDa subunit of the splicing factor U2AF (Valcarcel *et al*., 1993). hKIS binding was specific for C-terminal p27^{Kip1}

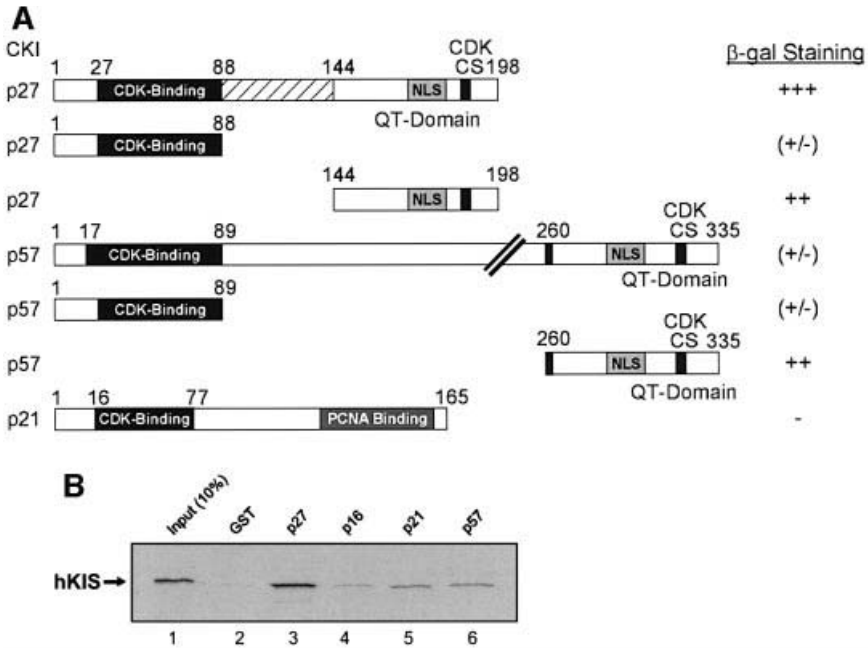


Fig. 1. Identification of hKIS and interaction with $p27^{Kip1}$. (**A**) hKIS interacts with the QT domain of $p27^{Kip1}$ in a yeast two-hybrid assay. hKIS was cotransfected into yeast with either $p27^{Kip1}$, $p27^{Kip1}$ [1–88 amino acids (aa)], $p27^{Kip1}$ (144–198 aa), $p57^{Kip2}$, $p57^{Kip2}$ (1–89 aa), $p57^{Kip2}$ (260–335 aa) or $p21^{Cip1}$. β-galactosidase assay on selection plates was performed and the intensity of staining was categorized. CDK CS, cyclin-dependent kinase consensus sequence; NLS, nuclear localization signal; PCNA, proliferating cell nuclear antigen. +++, very strong; ++, strong; (+/–), weak; –, no staining. (**B**) Interaction between hKIS and CKIs. hKIS is coimmunoprecipated with $p27^{Kip1}$, $p16^{Ink4}$, $p21^{Cip1}$ or $p57^{Kip2}$. GST indicates pGEX-6P backbone control. Input is [^{35}S]methionine-labelled hKIS control (10% of the total amount). Data are from an experiment that was repeated twice with similar results.

because it interacted poorly in the two-hybrid assay with N-terminal $p27^{Kip1}$, $p57^{Kip2}$, $p21^{Cip1}$ and several negative controls (Figure 1A and legend).

Human KIS was mapped using radiation hybridization to human chromosome 1q23.1, closest to marker SHGCOOH-36663. Northern blot analysis of multiple adult human tissues revealed a single 9.4 kb band in all tissues using full-length hKIS cDNA as a probe. The highest levels of hKIS mRNA expression were observed in skeletal muscle, kidney, placenta and peripheral blood leukocytes (Supplementary figure 1, available at *The EMBO Journal* Online).

hKIS interacts with $p27^{Kip1}$

To determine whether hKIS interacts directly with $p27^{Kip1}$ and other CKIs, glutathione *S*-transferase (GST) fusion proteins were incubated with labelled human hKIS generated by *in vitro* translation. hKIS directly bound GST–$p27^{Kip1}$ at levels significantly higher than $p21^{Cip1}$ or $p57^{Kip2}$ fusion proteins (Figure 1B, lane 3 compared with lanes 5 and 6), and binding of hKIS to $p16^{Ink4}$ was barely detectable (Figure 1B), suggesting specificity of hKIS binding to the Cip/Kip CKIs, predominantly $p27^{Kip1}$.

The kinase activity of hKIS was examined by incubation of *in vitro* translated and immunoprecipitated hKIS or a kinase-inactive hKIS mutant, K54A, with $p27^{Kip1}$. hKIS readily phosphorylated $p27^{Kip1}$, in contrast to the kinase inactive mutant K54A or a negative control (Figure 2A, lane 4 compared with lanes 2 and 3). hKIS and the K54A mutant were expressed at equivalent levels (data not shown). Under the same experimental conditions, hKIS did not phosphorylate $p16^{Ink4}$, $p21^{Cip1}$ or $p57^{Kip2}$ (data not shown), documenting the specificity of $p27^{Kip1}$ phosphorylation by hKIS. In addition, hKIS underwent autophosphorylation (Figure 2B, lanes 3, 5 and 7), as previously described for the rat homologue (Maucuer *et al*., 1997).

hKIS phosphorylates $p27^{Kip1}$ on S10

To determine the hKIS phosphorylation site on $p27^{Kip1}$, we generated additional GST fusion proteins and tested hKIS phosphorylation. We found that while hKIS bound C-terminal $p27^{Kip1}$, hKIS phosphorylated N-terminal $p27^{Kip1}$ and not C-terminal $p27^{Kip1}$ (Figure 2B, lane 7 compared with lane 5). Since hKIS did not phosphorylate C-terminal $p27^{Kip1}$, S178 and T187 were excluded as hKIS phosphorylation sites. To determine whether S10 was the putative phosphorylation site, mutational analyses of the N-terminal region were performed. Mutation of S10 to alanine [GST–p27(S10A)] abolished phosphorylation of GST–$p27^{Kip1}$ (Figure 2C, lane 4 compared with lane 2), indicating that hKIS phosphorylated $p27^{Kip1}$ on S10.

Next, we determined the two-dimensional (2D) phosphopeptide map of expressed $p27^{Kip1}$, p27(S10A) or p27(T187A) mutants following kinase assays with purified recombinant hKIS. Several radioactive spots were reproducibly detected, two of which (spots 1 and 2) appeared common to all maps. Two intensely labelled peptides (double arrows), however, were detected only in the maps of wild-type $p27^{Kip1}$ and the T187A mutant, but not in the map of the S10A mutant (Figure 3A), suggesting that these phosphopeptides contain S10 and confirming that hKIS phosphorylated $p27^{Kip1}$ on S10. The observation that the phosphopeptide containing S10 yielded two spots

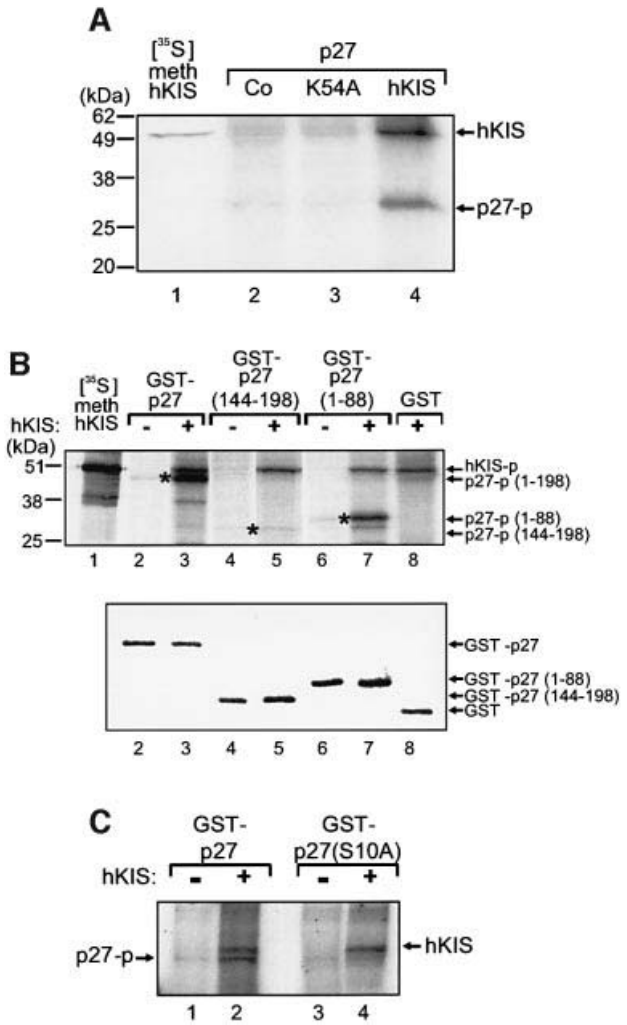


Fig. 2. Phosphorylation of CKIs by hKIS. (**A**) hKIS phosphorylates $p27^{Kip1}$. *In vitro*-transcribed and -translated hKIS was immunoprecipitated with Anti-Xpress antibody and incubated with GST-purified $p27^{Kip1}$. (Lane 1) [^{35}S]methionine-labelled hKIS control; (lane 2) immunoprecipitated pcDNA 3.1 Anti-Xpress tag backbone incubated with $p27^{Kip1}$ as a negative control; (lane 3) immunoprecipitated hKIS(K54A) mutant incubated with $p27^{Kip1}$; (lane 4) immunoprecipitated hKIS incubated with $p27^{Kip1}$. (**B**) hKIS phosphorylates $p27^{Kip1}$ at the N-terminal domain. N-terminal (codons 1–88) or C-terminal (codons 144–198) fragments of $p27^{Kip1}$ were purified as GST fusion proteins, and a kinase assay with hKIS was performed (upper panel). An equal amount of the GST fusion protein (40 pmol) was used in the kinase assay (upper panel), as stained by Coomassie Brilliant Blue (lower panel). (**C**) hKIS phosphorylates $p27^{Kip1}$ on S10. Serine 10 was substituted with alanine in a GST fusion protein $p27^{Kip1}$(S10A). The kinase assay was performed with hKIS (+, lanes 2 and 4) or a kinase-dead mutant, hKIS(K54A) (–, lanes 1 and 3).

is probably attributable to treatment during sample preparation.

It is possible that mutation of S to A might induce a change in the structure of $p27^{Kip1}$, which in turn may be responsible for the observed decrease in phosphorylation of the p27(S10A) mutant by hKIS. To determine whether hKIS phosphorylates $p27^{Kip1}$ on S10 directly, we prepared an antibody to a p27 S10 phosphopeptide (p27S10-p). The specificity of this antibody was analysed by western blot analysis and absorption tests. p27S10-p antibody was absorbed with the S10-p peptide (Figure 3B, lane 1), but not by the control protein, producing the expected 27 kDa band (Figure 3B, lane 2). Recombinant phosphorylated GST–$p27^{Kip1}$ protein was treated with calf intestinal alkaline phosphatase (CIAP) and probed with the p27S10-p antibody. Treatment with CIAP resulted in the disappearance of phosphorylated $p27^{Kip1}$ (Figure 3B, lane 4 compared with lane 3), and dephosphorylation of ^{32}P-labelled $p27^{Kip1}$ by CIAP was observed (Figure 3B, lane 8 compared with lane 7). To ensure that equal amounts of protein were used, the western blot was stripped and reprobed with a monoclonal $p27^{Kip1}$ antibody (Figure 3B, lanes 5 and 6). This specificity was confirmed further using ^{32}P-labelled $p27^{Kip1}$ (Figure 3B, lane 8 compared with lane 7). Specificity of the p27S10-p antibody was also confirmed in human embryonic kidney (HEK) 293 cells transfected with wild-type $p27^{Kip1}$ or p27(S10A) (Figure 3B, lanes 9 and 10).

hKIS phosphorylation of S10 was further confirmed with an *in vitro* kinase assay, incubating recombinant $p27^{Kip1}$ with or without hKIS. The addition of hKIS to the kinase assay resulted in S10 phosphorylation, detected by p27S10-p antibodies, while phosphorylated S10 was not detected in the absence of hKIS (Figure 3C, lane 2 compared with lane 1, upper panel), despite equal amounts of $p27^{Kip1}$ protein on the western blot (Figure 3C, lower panel). These findings provide additional evidence that hKIS phosphorylates $p27^{Kip1}$ on S10.

hKIS phosphorylates $p27^{Kip1}$ in vivo

We next examined whether hKIS phosphorylates $p27^{Kip1}$ *in vivo*. HEK 293 cells transiently expressing haemagglutinin (HA)-tagged $p27^{Kip1}$, p27(S10A), hKIS, or the hKIS kinase inactive mutant K54A were metabolically labelled with [^{32}P]orthophosphate and lysed. Recombinant $p27^{Kip1}$ protein was immunoprecipitated, subjected to SDS–PAGE and analysed by autoradiography. To ensure equivalent hKIS and $p27^{Kip1}$ protein levels, a western blot analysis was performed with hKIS and HA-tagged $p27^{Kip1}$ antibodies. hKIS phosphorylated $p27^{Kip1}$ (Figure 4, lane 3), but the hKIS kinase inactive mutant K54 did not (lane 5). Mutation of S10 prevented phosphorylation of $p27^{Kip1}$ by hKIS (Figure 4, lane 4). These data indicate that S10 is required for hKIS phosphorylation of $p27^{Kip1}$ *in vivo*.

Expression of endogenous hKIS

To investigate the expression and function of endogenous hKIS, polyclonal antibodies were raised in rabbit (hKIS 291 antibodies), and a monoclonal antibody (hKIS 3H2 antibody) was derived from mice. The specificity of both hKIS antibodies was confirmed by absorption using GST fusion proteins followed by western blot analysis of hKIS. The expected 49 kDa band was detected by western blotting with the hKIS 291 antibodies and 3H2 antibody in NIH 3T3 cells or primary smooth muscle cells (data not shown). Similar reactivity was observed in HEK 293 cells (Figure 5A, lanes 1 and 3, and B, lane 2). This band was not observed when the antibody was pre-absorbed with GST–hKIS (Figure 5A, lane 2, and B, lane 1).

We determined the subcellular localization of endogenous hKIS by immunofluorescence and confocal microscopy. In asynchronously growing NIH 3T3 cells, endogenous hKIS was detected mainly in the nucleus (Figure 5C, left lower panel). During serum starvation, nuclear hKIS expression was reduced (Figure 5C, left

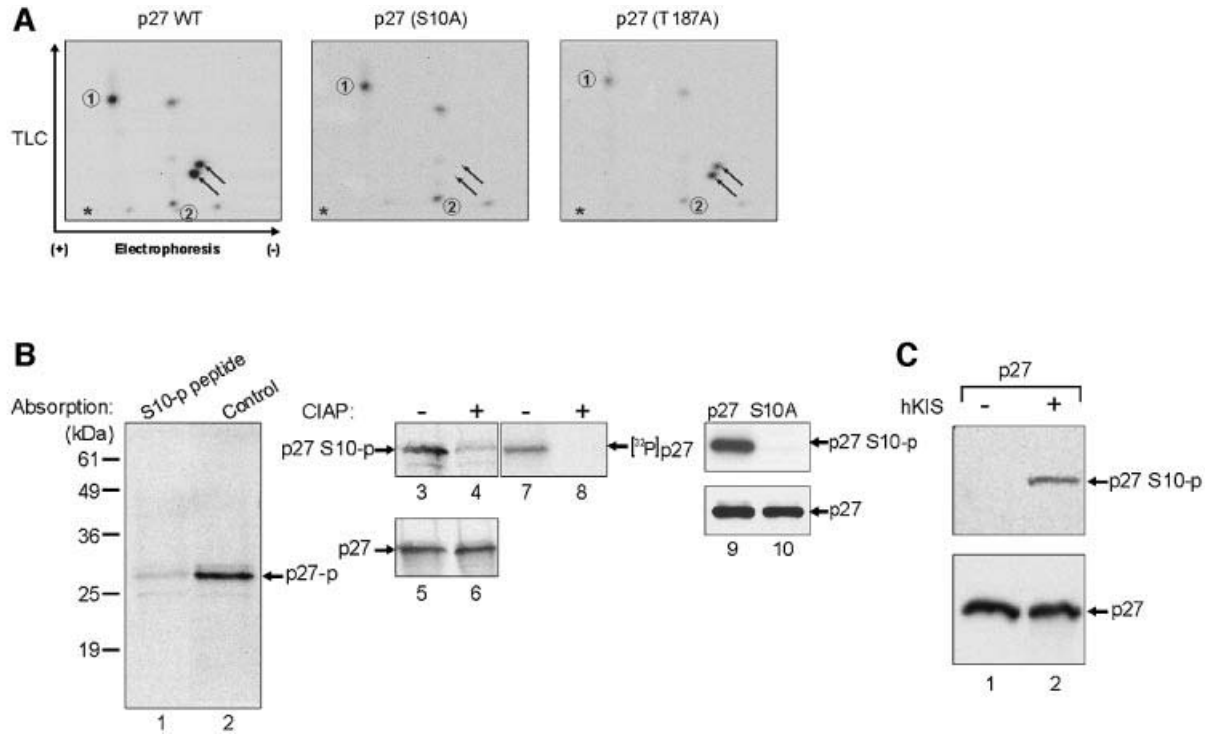


Fig. 3. hKIS phosphorylates p27^{Kip1} on S10. (**A**) Two-dimensional tryptic phosphopeptide mapping of wild-type and mutant p27^{Kip1}. p27^{Kip1}, p27(S10A) or p27(T187A) were expressed in HEK 293 cells and metabolically labelled with [^{32}P]orthophosphate in the presence of 50 μM lactacystin. The recombinant proteins were immunoprecipitated with p27^{Kip1} C19 antibody and subjected to 2D tryptic phosphopeptide mapping. Major phosphopeptides are numbered 1 and 2. Phosphopeptides containing S10 are indicated by arrows. An asterisk indicates the origin of migration, and arrows show the directions of separation by thin layer chromatography (TLC) and electrophoresis. (**B**) Mouse polyclonal antibodies recognize phosphorylated p27^{Kip1} on S10. A western blot of recombinant p27^{Kip1} phosphorylated by hKIS was performed with a p27S10-p antibody absorbed with non-phosphorylated recombinant p27^{Kip1} protein (Control, lane 2) or S10-p p27^{Kip1} peptide (lane 1). Phosphorylated p27^{Kip1} was incubated with (+) or without (–) CIAP, and a western blot with p27S10-p antibody was performed (lanes 3 and 4). The same blot was stripped and reprobed with a monoclonal p27^{Kip1} K25020 antibody (lanes 5 and 6). Recombinant p27^{Kip1} was phosphorylated by hKIS in the presence of [γ-^{32}P]ATP incubated with (+, lane 8) or without CIAP (–, lane 7). HEK 293 cell lysates were analysed on SDS–PAGE and a western blot was performed using the p27S10-p antibody (upper panel, lanes 9 and 10). The same blot was stripped and reprobed with a p27^{Kip1} C19 antibody (lower panel, lanes 9 and 10). (**C**) hKIS phosphorylates p27^{Kip1} on S10. Recombinant p27^{Kip1} protein was incubated with immunoprecipitated, *in vitro*-transcribed and -translated hKIS (+, lane 2), and a western blot with p27S10-p antibody was performed. The same blot was stripped and reprobed with a p27^{Kip1} K25020 antibody (lanes 1 and 2, lower panel).

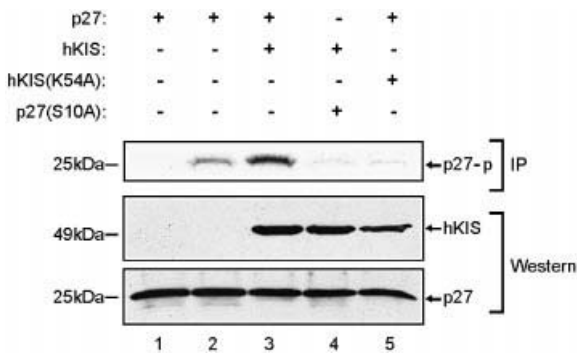


Fig. 4. hKIS phosphorylates p27^{Kip1} *in vivo*. HEK 293 cells transiently expressing HA-tagged wild-type p27^{Kip1}, hKIS, an hKIS(K54A) mutant or a p27(S10A) mutant were metabolically labelled with [^{32}P]orthophosphate. HA-tagged p27^{Kip1} and p27(S10A) proteins were immunoprecipitated with an HA antibody, subjected to SDS–PAGE and analysed by autoradiography. (Lane 1) a control immunoprecipitation using IgG; (lane 2) wild-type p27^{Kip1}; (lane 3) expression of wild-type p27^{Kip1} and hKIS; (lane 4) expression of p27^{Kip1}(S10A) and hKIS; (lane 5) expression of wild-type p27^{Kip1} and hKIS(K54A). A decrease in p27^{Kip1} protein might be expected following expression with hKIS (lane 3), but was not observed as the ratio of hKIS to p27^{Kip1} was 3:1.

upper panel). In contrast, endogenous p27^{Kip1} was expressed in the nucleus during serum starvation and shifted to the cytoplasm during serum stimulation (Figure 5C, p27, upper panel compared with lower panel). hKIS and p27^{Kip1} colocalize in the nucleus (Figure 5C, hKIS + p27). These findings demonstrate that endogenous hKIS is a nuclear protein and colocalizes with p27^{Kip1} in the nucleus during serum starvation. Furthermore, the data suggests that hKIS expression *in vivo* is regulated by serum growth factors.

Endogenous hKIS interacts with p27^{Kip1} in vivo

To investigate the interaction between endogenous p27^{Kip1} and hKIS, we examined mouse skin fibroblasts from p27^{Kip1} wild-type (p27$^{+/+}$) and null (p27$^{-/-}$) mice. hKIS coimmunoprecipitated with p27^{Kip1} in p27$^{+/+}$ cells (Figure 6, lane 3) but not in p27$^{-/-}$ cells (Figure 6, lane 2). The absence of p27^{Kip1} in null cells was confirmed by western blot [Figure 6, lane 4 compared with lane 5, p27^{Kip1} (p27) arrow], and comparable levels of KIS protein were present in p27$^{-/-}$ and p27$^{+/+}$ cells [Figure 6, lanes 4 and 5, hKIS (KIS) arrow]. In addition, p27^{Kip1} was detected in immunoprecipitates using hKIS 291 antibodies

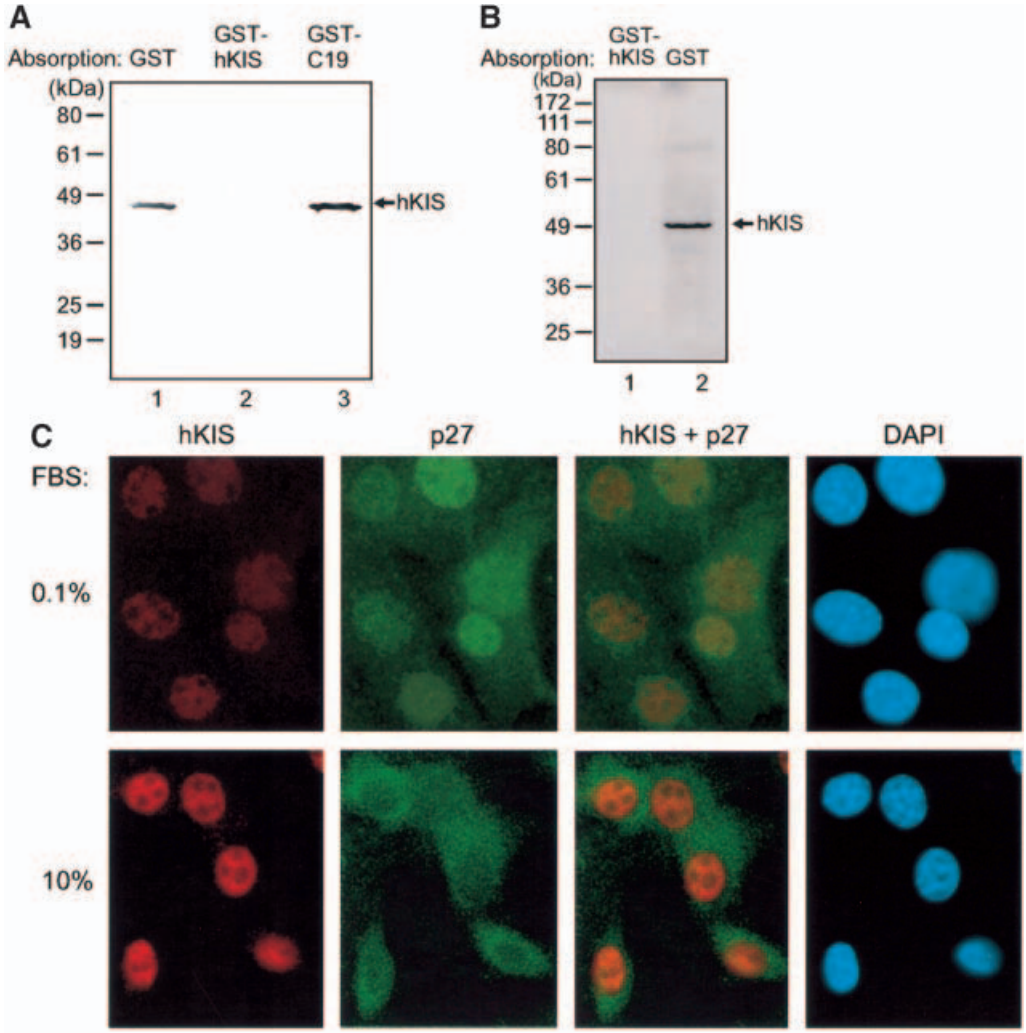


Fig. 5. hKIS is a growth factor-dependent kinase and localizes to the nucleus. (**A**) Specificity of polyclonal hKIS 291 antibodies. The antibodies were absorbed with GST fusion proteins, and immunoblotting was performed using NIH 3T3 lysates. (Lane 1) GST alone; (lane 2) hKIS blocked with GST–hKIS protein; (lane 3) an irrelevant GST fusion protein, GST–C19. (**B**) A mouse monoclonal 3H2 antibody recognizes hKIS. An immunoblot was performed using NIH 3T3 lysates after incubation of the mouse hKIS antibody with GST fusion proteins. (Lane 1) hKIS blocked with GST–hKIS protein; (lane 2) GST alone. (**C**) hKIS localizes to the nucleus. NIH 3T3 cells were serum starved for 36 h (0.1% FBS, upper panel), and then cells were serum stimulated for 6 h in 10% FBS (lower panel). Immunofluorescence and confocal microscopy were performed using hKIS 291 antibodies (left panel), a $p27^{Kip1}$ antibody K25020 (second panel from left) or both antibodies (third panel from left). A nuclear stain, DAPI, is shown on the far right panel.

but not with control IgG (Figure 6, lane 7 compared with lane 6). These data demonstrate that endogenous hKIS interacts with endogenous $p27^{Kip1}$ *in vivo*.

hKIS phosphorylation of $p27^{Kip1}$ on S10 in vivo is growth factor-dependent

To examine whether endogenous $p27^{Kip1}$ is phosphorylated by hKIS following serum stimulation, NIH 3T3 cells were serum starved for 36 h, followed by serum stimulation for 0–8 h. In these cells, hKIS kinase activity was present at a low level in G_0 cells, increased with serum stimulation, and was accompanied by an increase in hKIS protein levels (Figure 7A and B). In additional experiments, NIH 3T3 cells were serum starved for 36 h, followed by serum stimulation for 6 h in 10% fetal bovine serum (FBS). hKIS was expressed at ~5-fold higher levels during serum stimulation compared with serum starvation (data not shown).

Next, we performed 2D phosphopeptide mapping of endogenous $p27^{Kip1}$ following serum stimulation for 0–8 h. Following serum starvation, no radioactive labelled peptides were detected (Figure 7C, t_0). Following serum stimulation, two labelled peptides were weakly detected, and the intensity of these spots increased by 8 h (Figure 7C, arrows). The two intensely labelled peptides had a migration pattern that corresponded to the S10-containing phosphopeptides in Figure 3A. The findings suggested that S10 is a major phosphorylation site of endogenous $p27^{Kip1}$ and that serum stimulation increases hKIS kinase activity and $p27^{Kip1}$ S10 phosphorylation.

Phosphorylation of S10 by hKIS stabilizes $p27^{Kip1}$ in G_1

We examined the effect of hKIS phosphorylation on S10 on $p27^{Kip1}$ stability during G_1 in a pulse–chase analysis. Samples were taken at 0–8 h, and $p27^{Kip1}$ immunopreci-

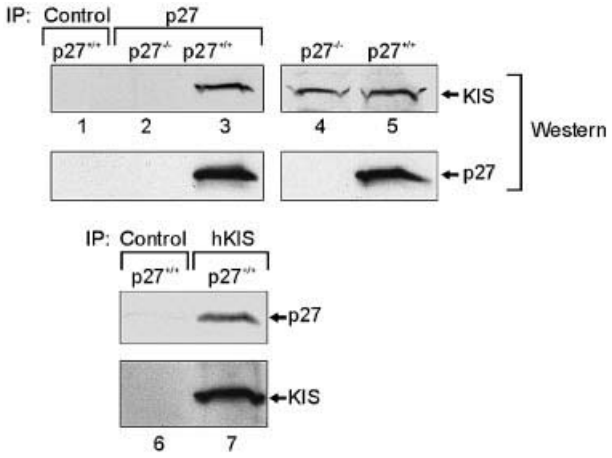


Fig. 6. hKIS kinase activity is growth factor-dependent and regulates $p27^{Kip1}$ *in vivo*. hKIS interacts with endogenous $p27^{Kip1}$. Fibroblasts from $p27^{Kip1}$ wild-type ($p27^{+/+}$) and null ($p27^{-/-}$) mice were incubated with AMP-PNP, $p27^{Kip1}$ was immunoprecipitated using $p27^{Kip1}$ C19 antibody, and a western blot was performed (lanes 2 and 3). hKIS and $p27^{Kip1}$ protein levels in $p27^{+/+}$ and $p27^{-/-}$ cells were determined using a western blot (lanes 4 and 5). To demonstrate a reciprocal $p27^{Kip1}$ and hKIS interaction, hKIS was immunoprecipitated with hKIS 291 antibodies, and a western blot was performed with $p27^{Kip1}$ K25020 antibody (lanes 6 and 7).

pitation was analysed. The expression of hKIS in cells stabilized $p27^{Kip1}$, compared with its absence (Figure 8A), with half-lives of 7.6 and 5.0 h, respectively (Figure 8B). The half-life of p27(S10A) was 4.2 h, in contrast to the phosphomimetic p27(S10D) mutant, which stabilized $p27^{Kip1}$ and prolonged its half-life to >8 h (Figure 8B). Inhibition of S10 phosphorylation by overexpression of the kinase-inactive mutant hKIS(K54A) decreased slightly the stability of endogenous p27, similar to the S10A mutant (data not shown). Our data are consistent with previous reports (Ishida *et al*., 2000; Rodier *et al*., 2001), and demonstrate that KIS phosphorylation of S10 stabilizes $p27^{Kip1}$ protein.

hKIS phosphorylation on S10 causes nuclear export of $p27^{Kip1}$

We reasoned that hKIS regulates the transport of $p27^{Kip1}$ and that the subcellular localization of $p27^{Kip1}$ controls its stability (Tomoda *et al*., 1999; Rodier *et al*., 2001). We examined the subcellular distribution of endogenous $p27^{Kip1}$ during cell cycle progression in NIH 3T3 cells expressing hKIS. In quiescent cells that expressed hKIS, $p27^{Kip1}$ was located in both the nucleus and the cytoplasm (Figure 9A and B). Serum stimulation caused a time-

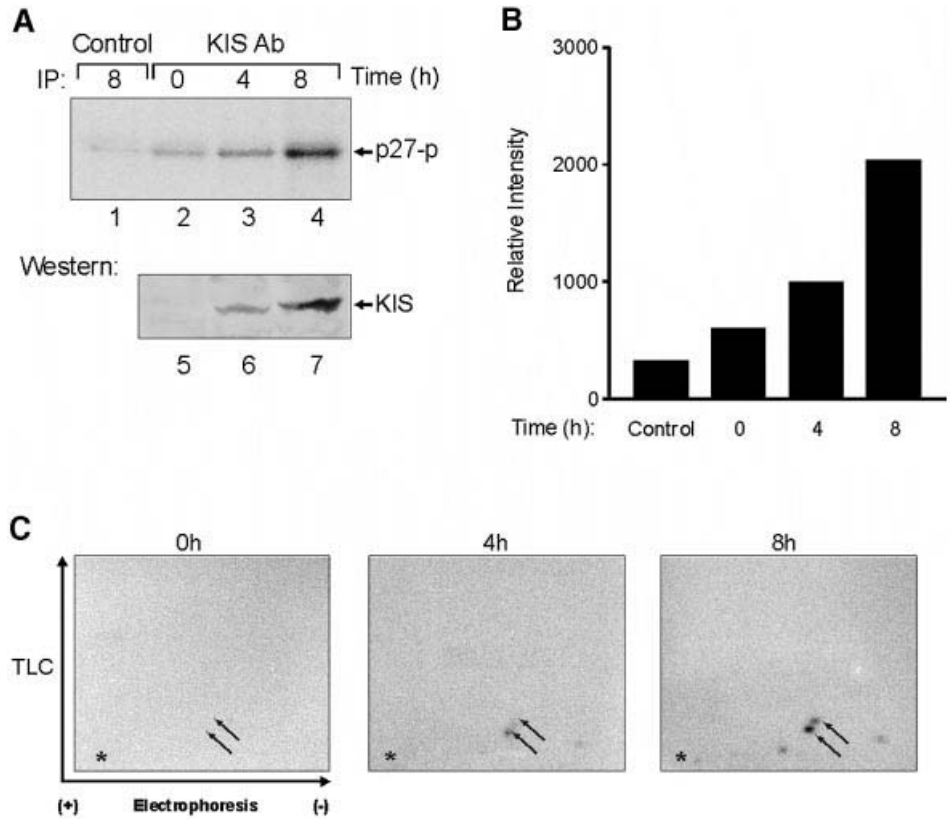


Fig. 7. hKIS kinase activity is associated with S10 phosphorylation of endogenous $p27^{Kip1}$. (**A**) KIS kinase activity increases following serum induction. NIH 3T3 cells were serum starved for 36 h, followed by serum stimulation for 0–8 h. hKIS protein was immunoprecipitated with hKIS 291 antibodies, and a kinase assay was performed with recombinant $p27^{Kip1}$ as a substrate (lanes 1–4, upper panel). hKIS protein levels were visualized by western blot analysis (lanes 5–7, lower panel). (**B**) Quantification of phosphorylated $p27^{Kip1}$ in NIH 3T3 cells treated with lactacystin. The relative intensity of phosphorylated recombinant $p27^{Kip1}$ by hKIS was determined at the indicated time points by densitometry (left). Cells were lysed at the indicated time points. Data are from an experiment that was repeated twice with similar results. (**C**) S10 is a major phosphorylation site following serum stimulation. Two-dimensional tryptic phosphopeptide mapping was performed on NIH 3T3 cells serum starved for 36 h, followed by serum stimulation for 0–8 h. Phosphopeptides containing S10 are indicated by arrows. An asterisk indicates the origin of migration, and arrows show the directions of separation by TLC and electrophoresis.

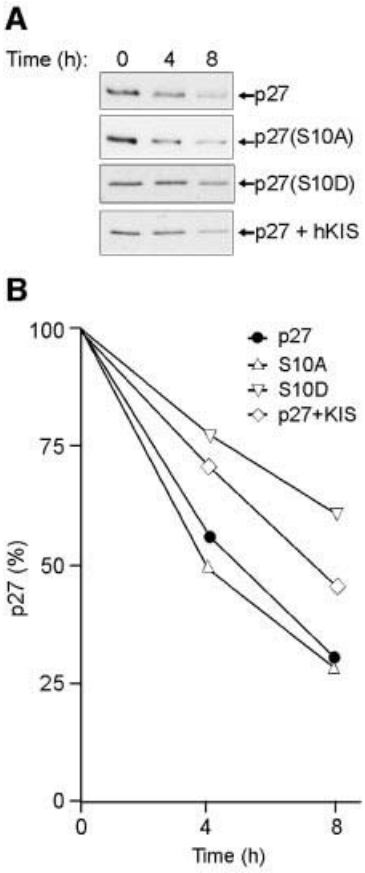


Fig. 8. hKIS stabilizes $p27^{Kip1}$ in G_1 cells. (**A**) NIH 3T3 cells were serum starved for 24 h and transfected with wild-type $p27^{Kip1}$, p27(S10A), p27(S10D), or $p27^{Kip1}$ and hKIS. The cells were pulse-labelled for 2 h with [^{35}S]methionine, and chased for the indicated time points in media containing 20% FBS. Cell lysates were immunoprecipitated with $p27^{Kip1}$ C19 antibodies, and the labelled $p27^{Kip1}$ protein was analysed by autoradiography. (**B**) Densitometric analysis of $p27^{Kip1}$ degradation rate. The intensity of the bands in (A) is expressed as a percentage of the time point t_0. Data are from an experiment that was repeated twice with similar results.

dependent redistribution of $p27^{Kip1}$ to the cytoplasm, with ~80% of cells showing cytoplasmic staining after 8 h. Treatment of cells with an inhibitor of nuclear export, leptomycin B (Nishi *et al*., 1994), prevented the cytoplasmic redistribution of $p27^{Kip1}$ after 8 h. Kinetic studies revealed that the cytoplasmic localization of $p27^{Kip1}$ occurred prior to activation of Cdk2 and most $p27^{Kip1}$ degradation (Figure 9C). These findings were confirmed in additional experiments by expressing p27-HA in NIH 3T3 cells and visualizing p27 localization with an HA antibody using immunofluorescence (see Supplementary figure 2) as shown previously (Rodier *et al*., 2001). Taken together, these data demonstrate that hKIS phosphorylation on S10 leads to nuclear export of $p27^{Kip1}$ to the cytoplasm.

hKIS promotes cell cycle progression

To determine whether hKIS phosphorylation of $p27^{Kip1}$ abolishes growth arrest, we measured the cell cycle distribution of HEK 293 cells expressing hKIS, $p27^{Kip1}$, the kinase inactive mutant hKIS(K54A), the S10A mutant $p27^{Kip1}$(S10A), the S10D mutant $p27^{Kip1}$(S10D), or $p21^{Cip1}$ alone or in different combinations by flow cytometry. $p27^{Kip1}$-transfected cells exhibited the expected G_1 cell cycle arrest (Figure 10A, $p27^{Kip1}$ compared with control), while hKIS reversed this inhibition. Overexpression of hKIS or hKIS(K54A) alone did not alter cell cycle distribution. Expression of $p27^{Kip1}$ with hKIS(K54A) also did not release the G_1 block, indicating that the functional effect of hKIS is dependent upon its kinase activity. The S10A mutant was more efficient than $p27^{Kip1}$ in causing cell cycle arrest, and this was not altered by coexpression with hKIS. The S10D mutant blocked the cell cycle less efficiently than wild-type $p27^{Kip1}$. Expression of hKIS with $p21^{Cip1}$ had no effect on $p21^{Cip1}$-induced cell cycle arrest, documenting the specificity of the interaction between hKIS and $p27^{Kip1}$ in cells. To exclude an effect of different expression levels, we measured the protein levels of the expressed vectors and found roughly comparable levels of protein (Figure 10B and C).

hKIS is required for S10 phosphorylation

These findings predicted that depletion of cellular KIS should lead to decreased phosphorylation of $p27^{Kip1}$ on S10 and growth arrest at G_1. To test this hypothesis, we used the small interfering RNA (siRNA) technique to reduce expression of KIS in HEK 293 cells (Zamore, 2001). Cells transfected with a double-stranded RNA (dsRNA) oligonucleotide for KIS showed reduced S10 phosphorylation compared with cells transfected with a control dsRNA oligonucleotide (Figure 11A and B), while levels of total $p27^{Kip1}$ protein remained unchanged (Figure 11B). Nuclear accumulation of $p27^{Kip1}$ corresponded to a gradual decrease in KIS levels (Figure 11C). Furthermore, in KIS siRNA cells, there was a greater accumulation of cells at the G_0/G_1 phase, in contrast to cells transfected with control oligos (Figure 11D). To determine whether $p27^{Kip1}$ is a critical target for KIS, $p27^{-/-}$ fibroblasts were transfected with KIS or control oligos. $p27^{-/-}$ cells treated with KIS siRNA oligos grew at similar rates and displayed comparable cell cycle progression compared with $p27^{-/-}$ cells treated with control oligos (Figure 11E). These results clearly demonstrate that hKIS is required for S10 phosphorylation *in vivo* and promotes cell cycle progression.

Discussion

Our data identify hKIS as the major kinase responsible for S10 phosphorylation on $p27^{Kip1}$. In the nucleus, hKIS binds the C-terminal of $p27^{Kip1}$ and phosphorylates the N-terminal on S10. Phosphorylation on S10 by hKIS causes nuclear export of $p27^{Kip1}$. The KIS kinase activity is induced by mitogens during G_0/G_1, where it promotes cell cycle progression. Depletion of hKIS using siRNA prevents S10 phosphorylation and leads to an accumulation of $p27^{Kip1}$ in the nucleus, enhancing growth arrest. $p27^{Kip1}$ is a critical target for KIS, as shown by siRNA experiments in which $p27^{-/-}$ cells treated with KIS siRNA behaved in a similar manner to control-treated $p27^{-/-}$ cells. Through its phosphorylation on S10, hKIS regulates the subcellular localization of $p27^{Kip1}$ and cell cycle progression in G_1 phase of the cell cycle.

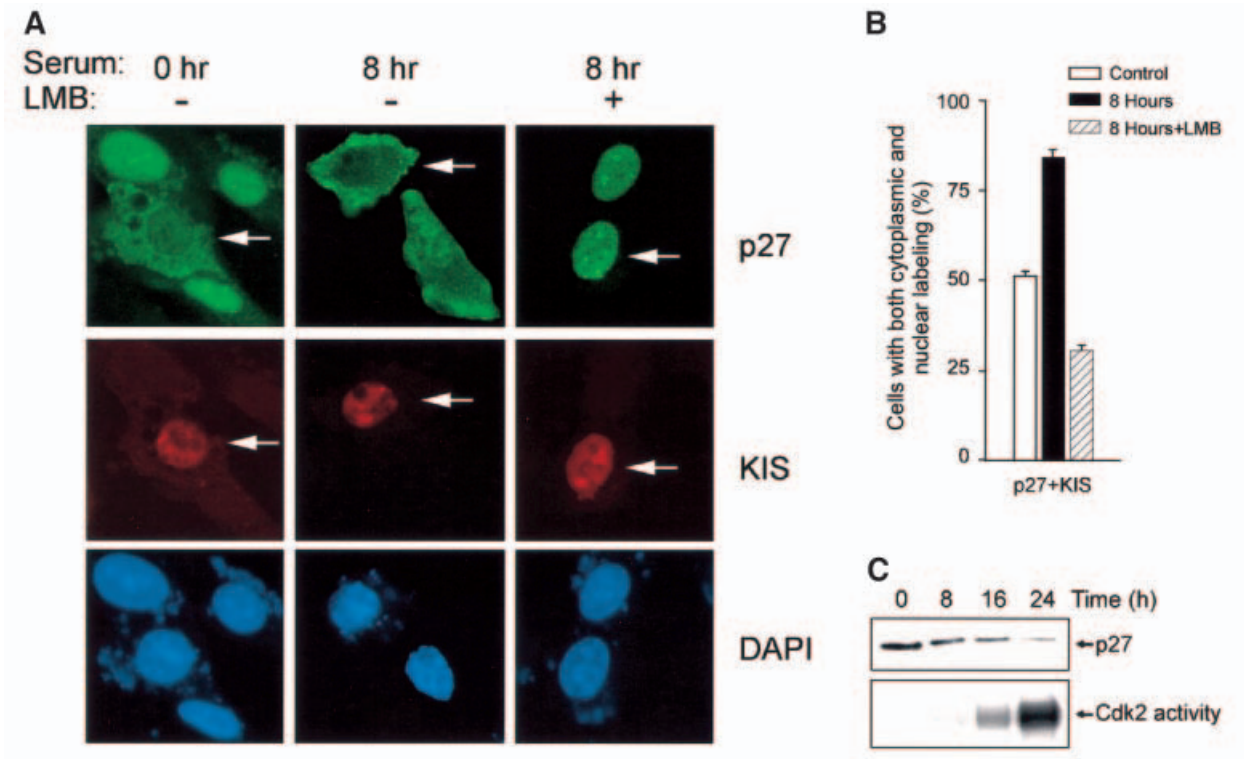


Fig. 9. hKIS phosphorylation on S10 causes nuclear export of p27Kip1. (**A**) Subcellular localization of endogenous p27Kip1 in NIH 3T3 cells transfected with hKIS. Cells were serum starved for 24 h, then serum stimulated for 8 h in the absence (–) or presence (+) of leptomycin B (LMB) 2 ng/ml, stained with p27Kip1 or hKIS antibodies, and examined by confocal microscopy. In serum-starved cells not expressing KIS, endogenous p27Kip1 is nuclear. Serum stimulation leads to redistribution to the cytoplasm. In contrast, in serum-starved cells expressing KIS, endogenous p27Kip1 is nuclear and cytoplasmic. After serum stimulation, endogenous 27Kip1 is cytoplasmic in these cells. The arrows indicate endogenous p27Kip1 in cells expressing KIS (upper panel) and transfected KIS (middle panel). A nuclear DAPI stain is shown in the lower panel. (**B**) Quantitative analysis of the cellular localization of endogenous p27Kip1 in cells transfected with hKIS. Results are expressed as the percentage of cells demonstrating both cytoplasmic and nuclear staining. Data are expressed as means ± SEM of three experiments. (**C**) Nuclear export precedes Cdk2 activation. Cells were serum starved for 24 h and stimulated with serum for the indicated times. p27Kip1 expression was examined by immunoblotting with p27Kip1 antibodies. Cdk2 activity was assayed using histone H1 as substrate.

The function of the KIS protein was previously unknown, having been defined initially by its phosphorylation of stathmin (Maucuer *et al*., 1997). Stathmin is a ubiquitous, cytosolic 19 kDa protein that is phosphorylated in response to growth and differentiation factors (Doye *et al*., 1990), neurotransmitters (Chneiweiss *et al*., 1992) and upon activation of T lymphocytes (Cooper *et al*., 1991), and thus stathmin has been proposed to function as a general integrator of signals controlling cell proliferation and differentiation. While KIS phosphorylates stathmin on serine residues, further analysis suggests that KIS phosphorylates synapsin and myelin basic protein *in vitro* on proline-directed residues (Maucuer *et al*., 2000). S10 is a putative target site for proline-directed kinases during the G_0/G_1 phases of the cell cycle. This site is conserved in mammalian p27Kip1 homologues but, interestingly, is not in related Cip/Kip proteins, p21Cip1 and p57Kip2, which may account for the specificity of KIS binding to p27Kip1 in preference to p21Cip1 and p57Kip2.

The activity of p27Kip1 is controlled by its abundance in different cellular compartments. In the nucleus, p27Kip1 exerts its inhibitory effect (Reynisdottir and Massague, 1997). The nuclear import of p27Kip1 is dependent upon a nuclear localization signal present in the C-terminal region of the protein and may require association with the nuclear pore-associated protein 60 (Muller *et al*., 2000). Our findings indicate that endogenous p27Kip1 is located in the nucleus and that p27Kip1 protein is translocated to the cytoplasm following phosphorylation of S10 by hKIS. hKIS kinase activity is present at a low level in G_0 cells and increases with serum stimulation. Endogenous p27Kip1 is efficiently transported to the cytoplasm in hKIS-expressing cells, and treatment of these cells with leptomycin B prevents the cytoplasmic relocalization of p27Kip1. Cytoplasmic redistribution of p27Kip1 is dependent upon the S10 residue as the p27(S10A) mutant remains in the nucleus, and the p27(S10D) mutant, which mimics phosphorylation, behaves similarly to hKIS and is efficiently exported to the cytoplasm. It is interesting to note that Meloche and colleagues found that phosphorylation of p27Kip1 on S10 occurs predominantly in G_0/G_1 cells, and that phosphorylation of S10 is not sufficient to induce nuclear export of p27Kip1, as the p27(S10D) mutant or wild-type p27Kip1 are not exported efficiently in G_0/G_1 cells, suggesting that a signal provided by growth factors or other proteins appears necessary to direct p27Kip1 export to the cytoplasm (Rodier *et al*., 2001). It is possible that KIS may provide this signal, in part, in addition to its role in S10 phosphorylation.

Phosphorylation of p27Kip1 on T187 by Cdk2 is thought to initiate the major pathway for p27Kip1 degradation (Pagano *et al*., 1995; Vlach *et al*., 1996). Cdk2 phos-

A

	G1-phase (%)	S-phase (%)	G2-phase (%)
Control	40.5+0.7	21.6+0.4	36.9+0.5
$p27^{Kip1}$	67.5+1.3	15.1+0.6	14.4+0.4
$p27^{Kip1}$+hKIS	45.9+0.9	19.0+0.5	34.0+1.0
$p27^{Kip1}$+hKIS(K54A)	70.4+0.7	13.1+0.1	16.2+0.6
$p27^{Kip1}$(S10A)	75.3+0.4	9.1+0.2	10.8+0.2
$p27^{Kip1}$(S10A)+hKIS	75.3+0.4	16.1+0.2	6.5+0.4
$p27^{Kip1}$(S10D)	51.4+0.3	19.8+0.1	28.2+0.1
$p21^{Cip1}$	64.5+0.4	17.4+0.1	17.8+0.4
$p21^{Cip1}$+hKIS	64.0+0.2	20.4+0.2	16.3+0.1
hKIS	40.3+0.8	21.9+1.0	36.7+0.7
hKIS(K54A)	53.0+0.2	14.4+0.1	31.7+0.5

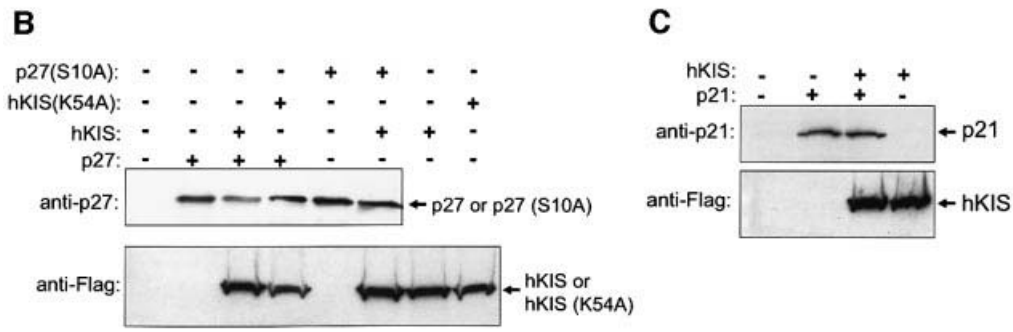


Fig. 10. hKIS promotes cell cycle progression. (**A**) hKIS releases G_1 arrest in $p27^{Kip1}$ cells. The vectors were expressed in HEK 293 cells with a CD2 reporter and analysed by propidium iodine staining of CD2-positive cells by FACS after 48 h. Experiments were performed in triplicate. Data are represented as means ± SEM. (**B** and **C**) hKIS downregulates $p27^{Kip1}$ protein levels, leading to cell cycle progression. The indicated vectors were expressed in HEK 293 cells, and cell lysates were analysed by western blotting using $p27^{Kip1}$ K25020 antibody for detection of $p27^{Kip1}$ and p27(S10A) (B, upper panel), a mouse anti-Flag antibody for hKIS and hKIS(K54A) (B, lower panel), and $p21^{Cip1}$ C19 antibodies for $p21^{Cip1}$ (C). Protein levels were measured by densitometry using an image analysis system (data not shown). Data are presented from an experiment that was repeated twice with similar results.

phorylation on T187 creates a binding site for SCF; ubiquitylation of $p27^{Kip1}$ results in degradation by the proteasome. Recently, a second proteolytic pathway for controlling $p27^{Kip1}$ that is activated by mitogens and degrades $p27^{Kip1}$ during G_1 has been described, and it has been proposed that the two proteolytic pathways act in sequence during the cell cycle to control $p27^{Kip1}$ abundance (Hara *et al*., 2001; Malek *et al*., 2001). Nuclear to cytoplasmic redistribution of $p27^{Kip1}$ may be an important component of the pathway in G_1. hKIS phosphorylation of $p27^{Kip1}$ on S10 during G_1 and the export of $p27^{Kip1}$ to the cytoplasm precede Cdk2 activation. Export of $p27^{Kip1}$ removes cyclin E and Cdk2 from nuclear targets and permits cell cycle progression through the G_1 checkpoint. Our observations that hKIS expression promotes cell cycle progression while depletion of hKIS by siRNA leads to G_1 arrest are consistent with this concept. Furthermore, S10 phosphorylation by hKIS resulting in nuclear export of $p27^{Kip1}$ may serve to lower the nuclear concentration of $p27^{Kip1}$ below a critical threshold, which would allow the activation of free cyclin E–Cdk2 (Rodier *et al*., 2001). Cytoplasmic localization of $p27^{Kip1}$ might also influence sequestration into cyclin D–Cdk complexes and contribution to the downregulation of $p27^{Kip1}$ in G_1. The relative contributions of the nuclear and cytoplasmic compartments of $p27^{Kip1}$ to degradation require additional study; however, downregulation of $p27^{Kip1}$ by hKIS phosphorylation on S10 in G_0/G_1 results may lead to the availability of cyclin E–Cdk2 complexes and the onset of the second proteolytic pathway operating in S and G_2 that is dependent upon phosphorylation of $p27^{Kip1}$ on T187 by Cdk2.

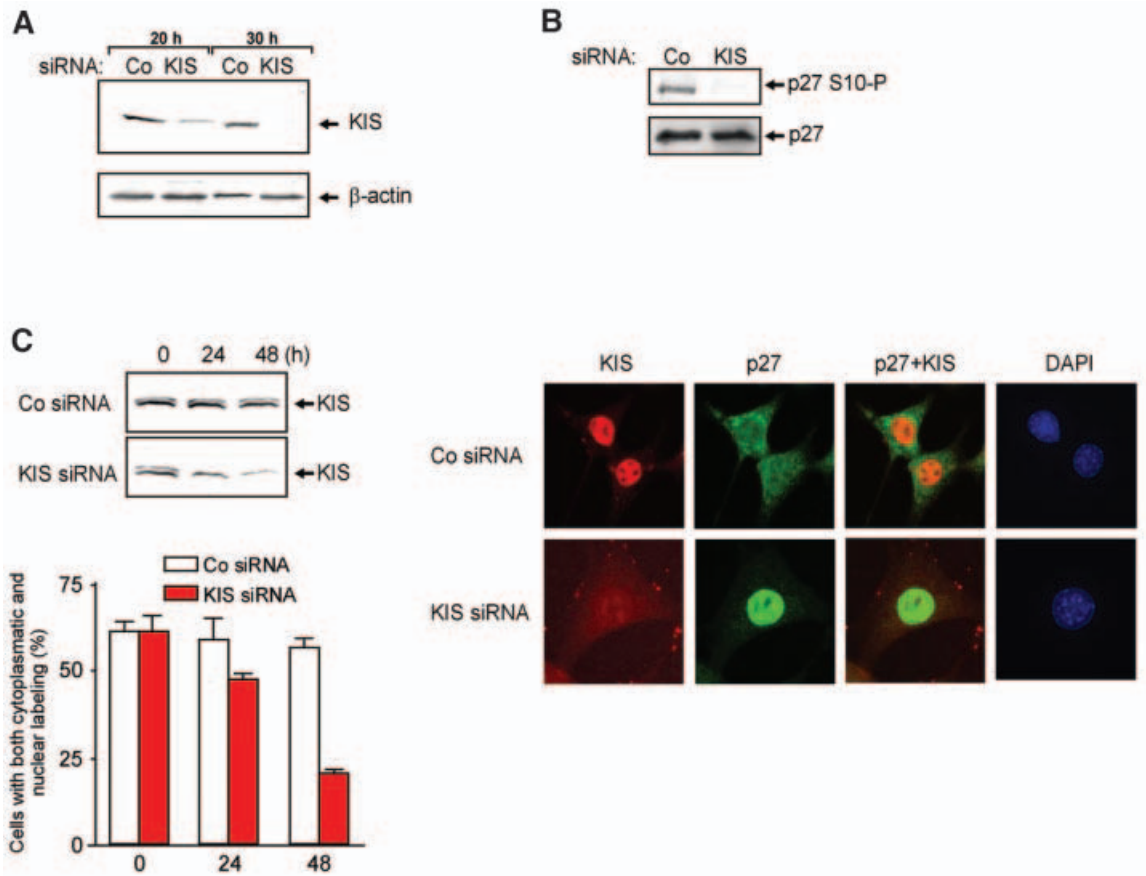
Materials and methods

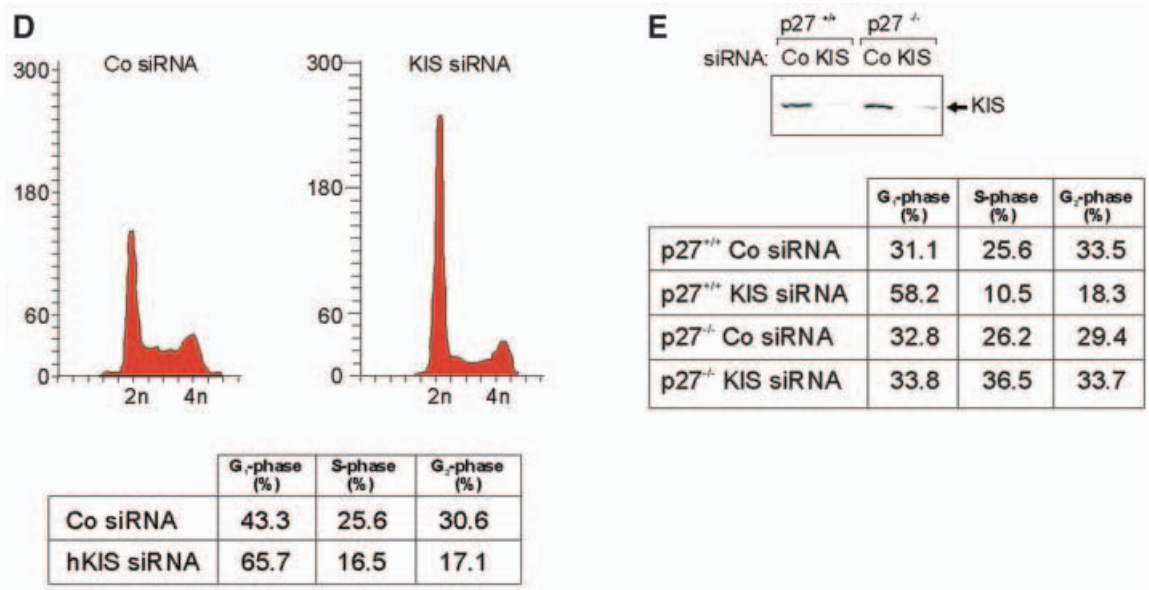
Yeast two-hybrid screen

A yeast two-hybrid screen was performed according to the Matchmaker Two-Hybrid system protocol (Clontech, Palo Alto, CA) using pGBT9$p27^{Kip1}$COOH as bait. Interactions were tested by direct cotransfection of GAL4 DNA-binding-domain-fused genes and the positive clones (fused to the GAL4 activating domain) into *Saccharomyces cerevisiae*. Sequence identification and comparisons were performed using the National Center for Biotechnology Information (NCBI) online service (http://www.ncbi.nlm.nih.gov; rat KIS accession No. X98374).

Vector construction

The expression vectors (see Supplementary data) were generated by insertion of the recombinant genes into pGBT9 (Clontech), pGEX-6P (Pharmacia, Piscataway, NJ) and pcDNA3.1/HIS (Invitrogen, Carlsbad, CA) (see Supplementary data). $p27^{Kip1}$(S10A), $p27^{Kip1}$(T187A), $p27^{Kip1}$(S10A/T187A) and hKIS(K54A) mutants were obtained by site-directed mutagenesis according to a standard protocol (Quikchange™; Stratagene, La Jolla, CA).





	G_1-phase (%)	S-phase (%)	G_2-phase (%)
Co siRNA	43.3	25.6	30.6
hKIS siRNA	65.7	16.5	17.1

	G_1-phase (%)	S-phase (%)	G_2-phase (%)
p27$^{+/+}$ Co siRNA	31.1	25.6	33.5
p27$^{+/+}$ KIS siRNA	58.2	10.5	18.3
p27$^{-/-}$ Co siRNA	32.8	26.2	29.4
p27$^{-/-}$ KIS siRNA	33.8	36.5	33.7

Fig. 11. hKIS is required for S10 phosphorylation. (**A**) Reduced hKIS in HEK 293 cells transfected with hKIS siRNA but not control siRNA. Cells were transfected with hKIS siRNA or control (Co) siRNA. At the indicated time points, cells were harvested and cell lysates were generated. Samples were immunoblotted with antibodies to hKIS. (**B**) Depletion of hKIS causes an absence of S10 phosphorylation. Cells were transfected as in (A). Six hours prior to lysis, cells were treated with lactacystin. Cells were harvested 30 h following transfection, and cell lysates were immunoblotted with a monoclonal p27 S10-p antibody (upper panel) or a monoclonal p27^{Kip1} antibody (lower panel). (**C**) hKIS is required for nuclear export of p27^{Kip1}. Cells were transfected with KIS siRNA or Co siRNA, harvested at 0, 24 or 48 h, and immunoblotting of cell lysates was performed with antibodies to KIS (upper left panel). Immunofluorescence for p27^{Kip1} was also performed, and the number of cells expressing cytoplasmic and nuclear p27^{Kip1} at 0, 24 and 48 h was counted (lower left panel). A minimum of 200 cells were scored. The results are expressed as the percentage of cells demonstrating both cytoplasmic and nuclear staining. Cells treated with Co or KIS siRNA and Co siRNA were immunostained for KIS and p27^{Kip1} + KIS 48 h after transfection, and examined by confocal microscopy (right panel). (**D**) Depletion of hKIS leads to G_1 arrest. Cells were transfected with Co or KIS siRNA, harvested at 30 h, and a FACS analysis was performed. (**E**) p27^{Kip1} is a critical target for KIS. p27$^{+/+}$ and p27$^{-/-}$ fibroblasts were treated with Co or KIS siRNA and harvested 48 h later. Cell lysates were immunoblotted with KIS antibodies (upper panel) and FACS analysis was performed (lower panel).

Protein production and in vitro binding

[^{35}S]methionine labelled and unlabelled hKIS were produced by *in vitro* transcription/translation using the T_NT T_7-coupled reticulocyte lysate system (Promega, Madison, WI) with pcDNA3.1KIS as template. The crude cell lysate of the GST-fused proteins was prepared according to the manufacturer's protocol (Pharmacia) and affinity-purified with glutathione–Sepharose 4B (Pharmacia). Binding assays were performed by incubation of GST fusion protein with 20 µl [^{35}S]methionine-labelled hKIS transcription/translation mixture at 4°C for 1 h in NP-40 buffer and washed three times. The bound proteins were analysed by SDS–PAGE. The quantity of GST fusion proteins was determined by comparison with a bovine serum albumin (BSA) standard on SDS–PAGE. To confirm the correct size and amount of GST fusion proteins, the polyacrylamide gel was stained with Coomassie Brilliant Blue R250 (Gibco, Gaithersburg, MD) prior to visualizing the [^{35}S]methionine-labelled hKIS by autoradiography using the Bio-Rad FX Image System (Bio-Rad, Hercules, CA). The GST moiety was cleaved from the fusion proteins using the PreScission™ protease (Pharmacia).

Northern blot and radiation hybridization

A human 12-lane multiple tissue northern blot and a human cancer cell line multiple-tissue northern blot (Clontech) were hybridized with ^{32}P-labelled hKIS cDNA. Radiation hybrid mapping was performed using Genebridge 3 radiation hybrid panel (Research Genetics, Huntsville, AL).

Cell culture and FACS

HEK 293 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% FBS, 2 mM glutamine plus antibiotics. Transfection of HEK 293 cells was performed at 40% confluence using Lipofectamine (Gibco) with 100 ng pVR1012p27^{Kip1}, pVR1012p21^{Cip1} and pVRCD2, and 5 µg pVR1012hKIS. Four hours after transfection the cells were split, and 36 h later FACS analysis was performed.

Western blot, immunoprecipitation and kinase assay

The following antibodies were used: a rabbit p27^{Kip1} antibody (C19; Santa Cruz Biotechnology, Santa Cruz, CA), a p27^{Kip1} mouse monoclonal K25020 (Transduction Laboratories, San Diego, CA), a rabbit p21^{Cip1} antibody (C19, Santa Cruz), a mouse monoclonal HA antibody (Roche, Indianapolis, IN), Flag antibody M2 (Sigma, St Louis, MO) and polyclonal hKIS 291 antibodies raised in rabbit immunized with DYLENEDEYEDVVEDVKEE MAP-peptide (Cocalico Biologicals, Inc., Reamstown, PA). The 291 antibodies were affinity purified using an AminoLink plus Immobilization kit (Pierce, Rockford, IL), using GST–hKIS as antigen. A hKIS 3H2 monoclonal antibody was raised in mice immunized with GST–hKIS (A&G Pharmaceutical, Inc., Baltimore, MD), and p27S10-p polyclonal antibodies were raised in mice immunized with -CNVRVSNG-pS-PSLE- peptide (Princeton Biomolecules, Langhorne, PA). Western blot and immunohistochemistry were performed as described previously (Yang *et al*., 1996). For detection of an association between endogenous hKIS and p27^{Kip1}, cells were incubated with 100 µM β-γ non-hydrolysable ATP analogue (AMP-PNP; Sigma) 4 h prior to lysis. Cell lysates (500 µg) were used for immunoprecipitation of p27^{Kip1}. *In vitro* kinase assays were performed using 20 µl of *in vitro* transcripted/translated hKIS. Kinase reactions were performed using purified hKIS kinase and GST-purified proteins.

Phosphopeptide mapping

Cells were ^{32}P-labelled as described previously (Gu *et al*., 1993). Labelled p27^{Kip1} was immunoprecipitated in RIPA using a rabbit p27^{Kip1} antibody (C19), and phosphopeptide mapping was performed using a Hunter Thin Layer Peptide Mapping Electrophoresis System (C.B.S., Del Mar, CA). Electrophoresis was performed using a low-pH buffer (50 ml formic acid, 156 ml acetic acid in 1794 ml deionized water pH 1.9) and phosphochromatography buffer (750 ml *N*-butanol, 500 ml pyridine, 150 ml acetic acid in 600 ml deionized water). Plates were air-dried and analysed using a Bio-Rad FX Imaging System.

Immunofluorescence and confocal microscopy

NIH 3T3 cells were grown on chamber slides (Lab-Tek; Nalge Nunc, Naperville, IL), serum starved for 36 h, serum stimulated for 6 h, fixed in 4% paraformaldehyde, and incubated with hKIS 291 antibodies or p27^{Kip1} K25020 antibody. Fluorescein isothiocyanate and rhodamine-conjugated secondary antibodies were used and mounted in DAPI-containing media.

Fluorescence emission images were obtained with a Zeiss confocal microscope system and collected with a C-Apochromat 63× (1.2 NA) water lens. For conventional fluorescence microscopy, samples were viewed using a fluorescence microscope (Nikon Eclipse E800). A minimum of 300 cells were scored for each coverslip. For experiments with leptomycin B, the drug was added at a final concentration of 2 ng/ml.

Pulse–chase assay

NIH 3T3 cells were serum starved with media containing 0.1% FBS for 24 h. At 12 h, cells were transfected using Lipofectamine 2000 (Invitrogen) and 10 h later metabolically labelled with Easy Tag Expression [^{35}S]protein labelling mix (NEN, Boston, MA) at a concentration of 100 µCi/ml for 2 h. After washing three times, the cells were incubated in isotope-free media containing 20% FBS for the indicated chase time.

siRNA

RNA interference was performed according to the manufacturer's protocol (Dharmacon Research, Lafayette, CO). Lipofectamine 2000 (Invitrogen) was used for transfection. dsRNAs corresponded to nucleotides 160 to 180 of the hKIS coding region (AAGCAGTTCTTGCCGCCAGGA) and the mouse KIS coding region (AAGCAGTTCCTGCCTCGGGGA). A mutated dsRNA (AAGCATTGCTTGACGCAAGGA; mutations underlined) and a non-related control dsRNA were used as described previously (Koepp *et al*., 2001).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank S.Elledge and E.Raines for reagents, and C.Coombs (NHLBI Light Microscopy Facility), K.Pearson, R.Weigert and C.E.Graham for technical assistance. M.B. was supported by a fellowship of the Deutsche Forschungsgemeinschaft.

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Received February 18, 2002; revised May 6, 2002; accepted May 7, 2002