A new pathway for mitogen-dependent Cdk2 regulation uncovered in p27^{Kip1}-deficient cells

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Background: The ability of cyclin-dependent kinases (CDKs) to promote cell proliferation is opposed by cyclin-dependent kinase inhibitors (CKIs), proteins that bind tightly to cyclin–CDK complexes and block the phosphorylation of exogenous substrates. Mice with targeted CKI gene deletions have only subtle proliferative abnormalities, however, and cells prepared from these mice seem remarkably normal when grown *in vitro*. One explanation may be the operation of compensatory pathways that control CDK activity and cell proliferation when normal pathways are inactivated. We have used mice lacking the CKIs p21^{Cip1} and p27^{Kip1} to investigate this issue, specifically with respect to CDK regulation by mitogens.

Results: We show that p27 is the major inhibitor of Cdk2 activity in mitogen-starved wild-type murine embryonic fibroblasts (MEFs). Nevertheless, inactivation of the cyclin E–Cdk2 complex in response to mitogen starvation occurs normally in MEFs that have a homozygous deletion of the *p27* gene. Moreover, CDK regulation by mitogens is also not affected by the absence of both p27 and p21. A titratable Cdk2 inhibitor compensates for the absence of both CKIs, and we identify this inhibitor as p130, a protein related to the retinoblastoma gene product Rb. Thus, cyclin E–Cdk2 kinase activity cannot be inhibited by mitogen starvation of MEFs that lack both p27 and p130. In addition, cell types that naturally express low amounts of p130, such as T lymphocytes, are completely dependent on p27 for regulation of the cyclin E–Cdk2 complex by mitogens.

Conclusions: Inhibition of Cdk2 activity in mitogen-starved fibroblasts is usually performed by the CKI p27, and to a minor extent by p21. Remarkably p130, a protein in the Rb family that is not related to either p21 or p27, will directly substitute for the CKIs and restore normal CDK regulation by mitogens in cells lacking both p27 and p21. This compensatory pathway may be important in settings in which CKIs are not expressed at standard levels, as is the case in many human tumors.

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Background

Cyclin-dependent kinases (CDKs) are not only essential for the cell division cycle but are also the targets of signals that regulate cell proliferation. This concept was first established genetically in fission yeast through studies of the relationship between cell size and cell division [1,2] and in budding yeast through studies of the cell cycle arrest induced by mating pheromone [3–6]. Other cues that modulate cell division have also been explained in terms of CDK regulation. The 'DNA-damage checkpoint' causes CDK inactivation [7–9] when the cell cycle is paused to allow completion of repair to damaged DNA and decreased CDK activity also accompanies exit from the cell cycle after mitogen starvation [10–12], after loss of integrin-dependent anchorage to a substratum [13–15], and during cellular differentiation [11,16–18]. In many cases, cells can be engineered to express CDK activity that is not sensitive to the anti-proliferative signal and this at least partially abrogates the cell cycle response as well, indicating that CDK downregulation is essential [13,16,19–22]. Thus, conditions that stop the cell division cycle often initiate pathways that lead to inactivation of CDKs.

CDK activity can be controlled by protein–protein interactions and by phosphorylation, and both of these general mechanisms have been implicated in CDK regulation in response to proliferative signals [23]. CDKs are positively regulated through their interactions with cyclins, and formation of the cyclin–CDK holoenzyme is upregulated by proliferative signals, such as serum mitogens [24], cell anchorage [25], and extracellular nutrients [26–28]. In some cases holoenzyme formation is accomplished by

modulating cyclin abundance through transcriptional [24,28–31] and translational pathways [25,27,32], and in others by controlling the assembly or the stability of the cyclin–CDK complex [33]. Knockout mice have been used to further refine this idea and to show that specific signaling pathways are linked to the regulation of individual cyclins. Thus, granulosa cell mitogenesis in response to follicle-stimulating hormone requires cyclin D2, and steroid-hormone-induced proliferation of the mammary epithelium requires upregulation of cyclin D1 [34,35].

Conversely, inhibition of CDK activity in response to antimitogenic signals can also be mediated by phosphorylation of residues within the ATP-binding pocket of the enzyme and by association of the CDK with inhibitory subunits. For instance, inhibitory phosphorylation of CDKs occurs as part of the 'budding checkpoint' pathway in *Saccharomyces cerevisiae* [36,37], and after DNA damage in a variety of organisms [8,21,38–41]. Binding of CDKs to inhibitory subunits called cyclin-dependent kinase inhibitors or CKIs [42] has been observed in response to contact inhibition, transforming growth factor β (TGF-β), mitogen starvation, loss of anchorage, cell senescence, DNA damage, and cell differentiation [42]. Experiments using immortalized mammalian cell lines have generally led to the conclusion that CKIs are necessary for these antimitogenic signals to stop the cell cycle. In mitogen-starved, quiescent murine and human cell lines p27 is upregulated and quantitatively associated with the cyclin E–Cdk2 complex. Antisense inhibition of p27 expression renders Cdk2 activity mitogen-independent, and delays cell cycle arrest after removal of serum mitogens [43,44]. Another example is targeted deletion of the gene encoding p21 from a colon carcinoma cell line, which abrogates the normal cell cycle arrest induced by DNA damage [45].

Mice with targeted deletions of specific CKI genes provide a different approach towards understanding the role of CKIs in controlling cell proliferation. These mice, and cells isolated from them, display subtle cell cycle abnormalities that are consistent with a role for the CKIs in regulating CDKs and cell proliferation. Mice that lack p21 have a partially defective cell cycle response to DNA damage [46–48], and keratinocytes isolated from these mice turn on differentiation markers *in vitro* more slowly than do wild-type cells [49]. Cells from p27-deficient mice show partially diminished mitogen-dependence both *in vitro* and *in vivo* [50–52]; this results in organ hypercellularity and mice that grow larger than normal. The $p57^{Kip2}$-deficient mice exhibit delayed or moderately impaired differentiation of cells in the chondrocyte and muscle lineages [53,54]. In none of these CKI-deficient mice, however, are there gross defects in cell differentiation, anchorage-dependence, contact inhibition, or responsiveness to TGF-β. This raises the question of whether there is functional redundancy among the CKIs or whether there might be other pathways for controlling cell cycle proteins that become operative when normal regulators are absent. Evidence for redundancy is provided by the synergistic enhancement of cell proliferation in the lens of mice lacking both p27 and p57 [55]. Nevertheless, even loss of both p27 and p57 had remarkably tissue-limited effects. We have pursued these questions by using genetically engineered mice lacking one or more CKIs to study CDK regulation by serum mitogens. We find that association with p27 is likely to be the primary means by which CDKs are inactivated in normal mitogen-starved cells, but unexpectedly that the Rb-related protein p130 can compensate for the absence of p27 and restore CDK regulation and mitogen-responsiveness to p27-deficient cells.

Results

Inactivation of cyclin E–Cdk2 in mitogen-starved cells

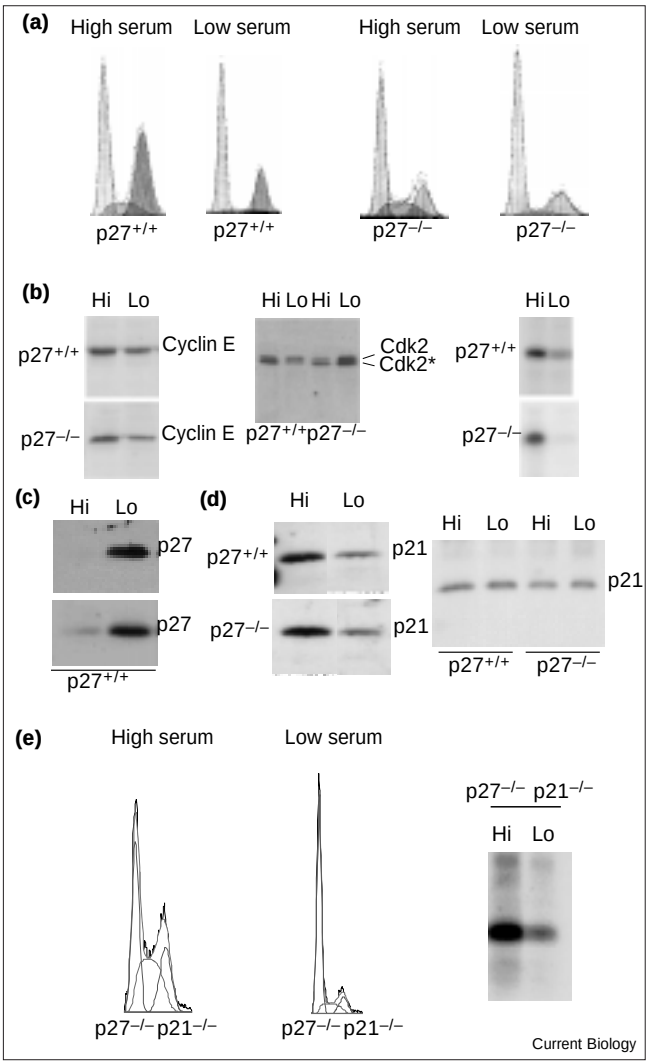
Murine embryonic fibroblasts (MEFs) were isolated from embryonic day 12 wild-type mice and maintained according to the 3T3 protocol. Wild-type MEFs underwent crisis approximately at passage 10 to 15, after which immortalized cell lines could be established. Cell cycle regulation was highly variable among different established lines, presumably reflecting the particular combination of mutagenic events that accompanied immortalization. Consequently the studies described below were performed exclusively on early passage (pre-crisis) cells, which behaved much more uniformly than immortalized cells.

Early passage 'primary' wild-type MEFs and MEFs containing a homozygous targeted deletion of the p27 gene (passage 2–4) [50] were placed into medium containing 0.1% serum for 24 hours and analyzed by flow cytometry. MEFs of both genotypes efficiently underwent growth arrest at the G0–G1 phase boundary following serum starvation (Figure 1a). The expression of neither the gene encoding cyclin E (Figure 1b) nor that encoding Cdk2 (data not shown) was significantly decreased in quiescent MEFs, and the abundance of the cyclin E–Cdk2 complex closely approximated that in proliferating cells (Figure 1b); nevertheless, in both $p27^{+/+}$ and $p27^{-/-}$ MEFs cyclin E–Cdk2 complexes were catalytically active only in proliferating cells (Figure 1b). Cyclin E and Cdk2 were both located in the nucleus in quiescent MEFs (data not shown). The mechanism by which Cdk2 activity is downregulated following mitogen starvation in wild-type and p27-null MEFs is the subject of this study.

Regulation of cyclin E–Cdk2 by the p27 and p21 CKIs

In primary wild-type MEFs the CKI p27 was induced by mitogen starvation (Figure 1c) whereas the level of p21 slightly declined (Figure 1d). Significant amounts of p57 were not detected (data not shown). The association of cyclin E and Cdk2 with p21 and p27 was measured by immunoprecipitation and by western blotting (Figure 1c,d). In wild-type MEFs the association of cyclin E and Cdk2

Figure 1



Regulation of cyclin E–Cdk2 in wild-type and $p27^{-/-}$ MEFs. (a) Wild-type ($p27^{+/+}$) and $p27^{-/-}$ proliferating MEFs (high serum) were placed in 0.1% serum for 24 h (low serum) and subsequently analyzed by flow cytometry. (b) Extracts were prepared from proliferating (high serum, Hi) or serum-starved (low serum, Lo) MEFs and were immunoblotted with cyclin-E-specific antiserum (left). These same extracts were immunoprecipitated with cyclin E antibody. The amount of Cdk2 bound to cyclin E was determined by western blot (middle); Cdk2* designates the phosphorylated, active form of Cdk2. The amount of cyclin-E-associated histone H1 kinase activity was measured (right). In this experiment, and in all other cyclin E immunoprecipitations, excess anti-cyclin E antibody was used and it was confirmed that cyclin E was quantitatively immunoprecipitated from the cell extracts. (c) The amount of p27 expressed in proliferating and serum-starved MEFs was determined by western blot. The top panel is a p27 western blot of the whole-cell extract, and the bottom panel is a western blot of cyclin E immunoprecipitates. (d) The amount of p21 expressed in proliferating and serum-starved MEFs was determined by western blot (left). The amount of p21 bound to cyclin E was measured in the same samples (right). (e) Flow cytometry was performed on proliferating (high serum) or serum-starved (low serum) primary $p27^{-/-}p21^{-/-}$ MEFs. Cyclin E was immunoprecipitated from these cells and the amount of associated kinase activity measured using histone H1 as a substrate (right).

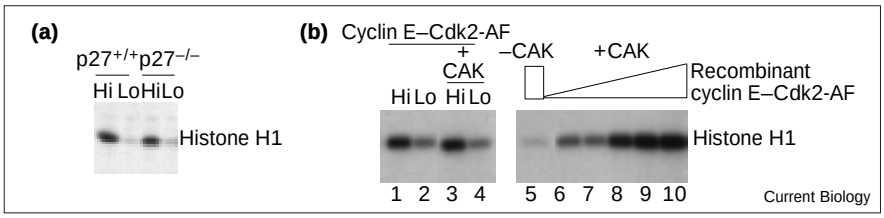
with p27 greatly increased in serum-starved cells, whereas the association of cyclin E–Cdk2 with p21 was essentially unchanged. Immunodepletion of p27 from lysates of quiescent cells removed at least 90% of the cyclin E–Cdk2 complexes, whereas most cyclin E–Cdk2 complexes in proliferating cells were not associated with p27 (data not shown). Together, these studies suggest that induction of p27 and the binding of p27 to cyclin E–Cdk2 results in Cdk2 inactivation in serum- starved MEFs, as previously observed in established murine cell lines [44]. The data also allow for the possibility that p21 participates in Cdk2 inactivation in mitogen- starved cells, but its contribution would be relatively minor.

Even though p27 is the major inhibitor of cyclin E–Cdk2 in quiescent wild-type MEFs, cyclin E–Cdk2 complexes isolated from quiescent $p27^{-/-}$ MEFs were nonetheless catalytically inactive (Figure 1b). Hence, other inhibitors must substitute for p27. Whatever the compensatory pathway is, it does not solely involve p21, because p21 levels were not elevated in $p27^{-/-}$ cells when compared with wild-type MEFs, nor was there a change in the amount of p21 bound to cyclin E (Figure 1d). To further substantiate this conclusion MEFs were prepared from mice containing homozygous deletions of both the p21 and p27 genes. MEFs taken from $p21^{-/-}p27^{-/-}$ doubleknockout mice exited from the cell cycle following serum starvation as effectively as did wild-type cells (Figure 1e). When compared with proliferating cells, serum-starved $p21^{-/-}p27^{-/-}$ MEFs had a slight decrease in the levels of cyclin E protein. Nevertheless, the amount of Cdk2 that associated with cyclin E was equivalent between proliferating and mitogen-deprived cells, and both the Thr160- phosphorylated and unphosphorylated forms of Cdk2 were present in the complex (data not shown). The cyclin E–Cdk2 complex was catalytically inactive in serum- starved $p21^{-/-}p27^{-/-}$ cells, however, and therefore was mitogen-responsive despite the absence of both p21 and p27 (Figure 1e).

No evidence for altered Cdk2 phosphorylation in p27-null MEFs

Inhibitory phosphorylation of Cdk2 on Thr14 and/or Tyr15 might have accounted for its downregulation in quiescent $p27^{-/-}$ cells. To test this hypothesis, $p27^{-/-}$ cells were transfected with a mutant version of Cdk2 (Cdk2-AF) in which both Thr14 and Tyr15 were replaced with nonphosphorylatable residues (Thr14 to alanine and Tyr15 to phenylalanine). Cells were transfected with plasmids encoding Myc-epitope-tagged cyclin E (Myc–cycE) and haemagglutinin(HA)-epitope-tagged Cdk2-AF (HA–Cdk2-AF), and then serum-starved for 24 hours. The catalytic activity of the cyclin E–Cdk2-AF complex was measured following immunoprecipitation using antibodies specific for human cyclin E (Figure 2) or HA (data not shown) epitopes. Figure 2a shows that cyclin E–Cdk2-AF complexes were inactive in serum-starved $p27^{-/-}$ and $p27^{+/+}$ MEFs,

Figure 2



Phosphorylation of Cdk2 does not regulate its activity in quiescent p27$^{-/-}$ MEFs. The p27$^{-/-}$ and p27$^{+/+}$ MEFs were transfected with Myc-epitope-tagged human cyclin E and an HA-epitope-tagged Cdk2 mutant (Cdk2-AF) that replaced Thr14 with an alanine and Tyr15 with phenylalanine. Transfected cells were re-fed with medium containing 10% serum (Hi) or 0.1% serum (Lo) and incubated for 24 h. (a) Immunoprecipitations were performed using an anti-HA antibody and the immunoprecipitates assayed for kinase activity against histone H1. (b) Immunoprecipitations were performed as in (a) except that antibodies that recognize the transfected cyclin E were used instead of anti-HA. The immunoprecipitations were divided and half of the cyclin E immunoprecipitate was left untreated and half was treated with active recombinant CAK. Control experiments were done to adjust the amount of CAK in order to ensure that it was not a limiting component of the reactions (data not shown). The titration of the recombinant cyclin E–Cdk2 complexes was adjusted so that the amount of cyclin E–Cdk2 immunoprecipitated from the cell extract was at the midpoint of the titration (data not shown). Histone H1 kinase assays were performed on cyclin E immunoprecipitates (lanes 1–2) or on CAK-treated samples (lanes 3–4). For comparison, a titration (1–5 μl) of inactive recombinant cyclin E–Cdk2 (HA-tagged) complexes were immunoprecipitated with human-specific anti-cyclin E antibodies and incubated with a fixed amount of active CAK. Histone H1 kinase assays were performed on CAK-activated recombinant cyclin E–Cdk2 complexes (lanes 6–10) and control untreated immunoprecipitated cyclin E–Cdk2 complexes (lane 5).

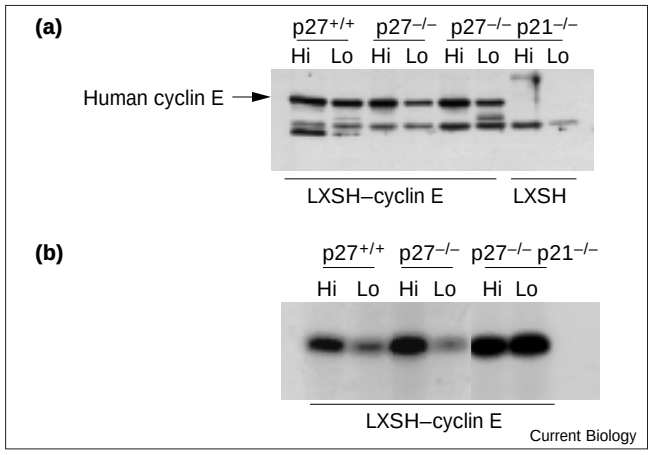
demonstrating that phosphorylation at Thr14 or Tyr15 is not necessary for inhibiting cyclin E–Cdk2 kinase activity.

An alternative explanation for inactivation of the cyclin E–Cdk2 complex in p27$^{-/-}$ cells was downregulation of the CDK activating kinase (CAK) and therefore a lack of phosphorylation at Thr160. To address this issue, we tested whether recombinant CAK could activate cyclin E–Cdk2-AF complexes that had been immunoprecipitated from serum-starved p27$^{-/-}$ cells (Figure 2). As a control we used immunoprecipitated, inactive, recombinant cyclin E–Cdk2 complexes that had been assembled *in vitro* by mixing extracts of insect cells that had been separately infected with cyclin E and Cdk2 baculoviral expression vectors. In a side-by-side experiment, CAK did activate recombinant, inactive cyclin E–Cdk2 complexes but did not activate cyclin E–Cdk2 complexes isolated from serum-deprived p27$^{-/-}$ MEFs. This experiment was performed within the linear range of the assay, and an equal amount of cyclin E–Cdk2-AF and recombinant cyclin E–Cdk2 complexes were used. In sum, the inactivation of cyclin E–Cdk2 complexes in mitogen-deprived p27$^{-/-}$ MEFs could not be explained by either the presence of an inactivating phosphorylation at Thr14 and Tyr15 or the absence of CAK.

A titratable Cdk2 inhibitor in p27$^{-/-}$ cells

The above experiments indicated that none of the commonly studied mechanisms was responsible for downregulating Cdk2 in serum-starved p27$^{-/-}$ cells. To determine whether or not a stoichiometric inhibitor of cyclin E–Cdk2 might compensate for the absence of p27, we examined the effect of increasing the amount of cyclin E–Cdk2 complexes in quiescent cells. We established stable cell lines that overexpressed human cyclin E about threefold to fourfold compared with the endogenous murine protein (Figure 3a, and data not shown). Control cell lines infected with the LXSH viral vector alone were also established. Overexpression of cyclin E did not block the exit from the cell cycle in serum-starved wild-type, or p27$^{-/-}$ MEFs (Table 1). In contrast, overexpression of cyclin E in cells lacking both p21 and p27 stimulated cells to remain in the cell cycle for at least 24 hours following serum deprivation (Table 1). Moreover, p21$^{-/-}$p27$^{-/-}$ MEFs had increased levels of cyclin-E-associated kinase activity in both proliferating and serum-starved cells when compared

Figure 3



A titratable inhibitor of cyclin E–Cdk2 in p21$^{-/-}$p27$^{-/-}$ MEFs. (a) Western blots were performed using antibodies that specifically recognize human cyclin E on proliferating (high serum, Hi) and serum-starved (low serum, Lo) MEFs of the indicated genotypes that had been infected with a retrovirus expressing human cyclin E (LXSH–cyclin E) or empty retroviral vector (LXSH). (b) Histone H1 kinase assays were performed on human-specific cyclin E immunoprecipitations from the cells characterized in (a).

Table 1

Cell cycle analysis of cyclin E overexpressors.

		% Flow cytometry profile		
Cell genotypes	% Serum	G1	S phase	G2/M
$p27^{+/+}$	10	64.2	19.6	16.1
	0.1	70.6	6.2	23.1
$p27^{-/-}$	10	60.6	24.6	23.1
	0.1	86.9	5.3	7.6
$p21^{-/-}p27^{-/-}$	10	43.7	41.9	14.2
	0.1	53.6	30.5	15.8

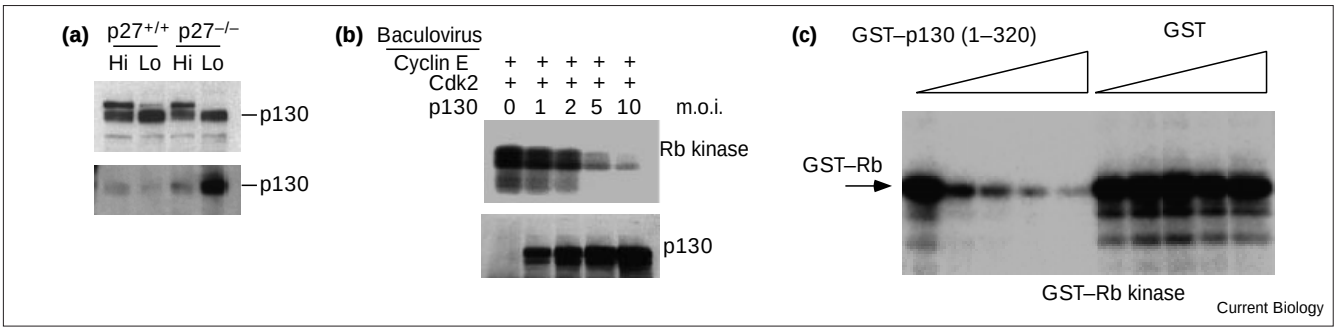
with wild-type or $p27^{-/-}$ cells (Figure 3b). These effects are not due to differences in the amount of exogenous cyclin E expression between cells of various genotypes (Figure 3a). In the absence of both p27 and p21, therefore, overexpression of cyclin E results in constitutive cyclin-E-associated kinase activity and in dysregulation of serum responsiveness. These results showed that the inhibition of Cdk2 kinase activity in serum-starved p27-deficient cells is due to a titratable inhibitor. This inhibitor was not heat stable (data not shown).

Inhibition of Cdk2 by p130

Previous work has shown that binding of the Rb-related protein p107 to the cyclin E–Cdk2 complex inhibits the phosphorylation of exogenous substrates [15]. Although p107 is expressed primarily in proliferating cells, another Rb family member, p130, is induced in quiescent cells [56,57]. Moreover, recent work has shown that p130, like p107, can inhibit CDKs *in vitro* [58], but neither p130 nor p107 has previously been implicated in the physiological regulation of CDK catalytic activity *in vivo*. MEFs, like other previously studied cell types, induce p130 when starved of serum mitogens (Figure 4a; note that the upper band is non-specific as it is also present in p130-null MEFs; see later). In wild-type cells only a small fraction of cyclin E is bound to p130 (Figure 4a), probably because cyclin E is instead tightly bound to p27 in quiescent cells and to p21 in proliferating cells. In $p27^{-/-}$ cells the association of p130 with both cyclin E and Cdk2 is altered. First, a substantial amount of p130 is bound to cyclin E specifically in quiescent $p27^{-/-}$ cells (Figure 4a). Moreover, immunodepletion experiments show that in quiescent cells lacking both p27 and p21 more than 80% of cyclin E–Cdk2 complexes are bound to p130 (data not shown). Second, the association of p130 with Cdk2 is increased in both dividing and non-dividing cells (data not shown). In dividing cells this presumably represents association of p130 with a fraction of the cyclin A–Cdk2 complexes, which are much more abundant than cyclin E–Cdk2.

We also showed that the binding of p130 to cyclin E–Cdk2 inhibits its catalytic activity both *in vivo* and *in vitro*. Insect cells were co-infected with baculoviral vectors expressing cyclin E and Cdk2, together with an increasing amount of a baculoviral vector expressing p130. Cyclin E–Cdk2 complexes were then immunoprecipitated and their activity measured by phosphorylation of the Rb protein. In a dose-dependent manner, p130 inhibited cyclin E–Cdk2 protein kinase activity (Figure 4b). Similarly, recombinant p130 coupled to glutathione-S-transferase (GST) purified from *Escherichia coli* inhibited cyclin

Figure 4



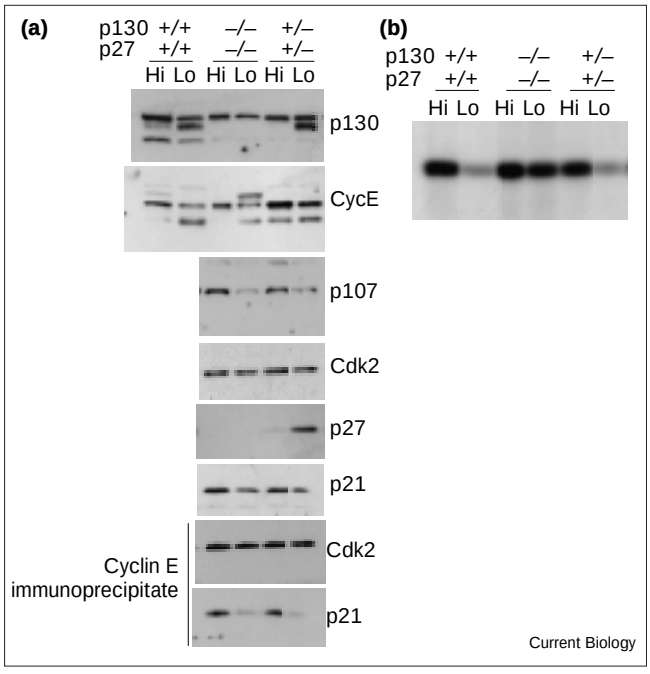
The p130 protein binds to and inactivates cyclin E–Cdk2 complexes *in vivo* and *in vitro*. (a) Proliferating (high serum, Hi) $p27^{+/+}$ and $p27^{-/-}$ MEFs were placed in 0.1% serum for 24 h (low serum, Lo); p130 western blots were then performed on whole-cell extracts (top) and cyclin E immunoprecipitates (bottom). (b) Insect cells were infected with equal amounts of human cyclin E and Cdk2 virus and at the indicated multiplicity of infection (m.o.i.) for a p130-expressing virus (top). Extracts from infected cells were immunoprecipitated using a human cyclin-E-specific monoclonal antibody and Rb kinase assays were performed (bottom). Then p130 western blots were performed on extracts derived from infected cells. Cyclin E and Cdk2 expression, as well as cyclin E–Cdk2 assembly, were monitored and shown not to be affected by co-expression of p130 (data not shown). (c) Cyclin E was immunoprecipitated from insect cells infected with equivalent amounts of cyclin E and Cdk2 virus. Rb kinase assays were performed on immunoprecipitated recombinant active cyclin E–Cdk2 complexes that were incubated with a titration (0, 1.25, 2.5, 3.75 or 5 μg) of bacterially expressed purified GST alone or GST–p130 (amino acids 1–320).

E–Cdk2 activity *in vitro*, showing that this was a property of p130 alone and did not require a p130-associated cellular protein (Figure 4c). Moreover, this latter experiment used a portion of p130 (residues 1–320) that does not contain a Cdk2 phosphorylation site demonstrating that inhibition of Rb phosphorylation *in vitro* is not due to substrate competition.

Deregulation of cyclin E–Cdk2 in p27$^{-/-}$p130$^{-/-}$ cells

The essential role of p130 in Cdk2 regulation was confirmed by examining cyclin E–Cdk2 activity in MEFs lacking both p27 and p130. When compared with control MEFs harvested from littermates that were heterozygous at both loci (p27$^{+/-}$p130$^{+/-}$), the doubly deficient MEFs (p27$^{-/-}$p130$^{-/-}$) expressed equal amounts of cyclin E, Cdk2, p21, cyclin E–Cdk2 complexes and cyclin E–p21 complexes, either in proliferating or serum-starved cells (Figure 5a). Also, there was no evidence for a compensatory increase in p107 expression in the p130-null MEFs (Figure 5a). Cyclin E–Cdk2 kinase activity was downregulated by mitogen starvation in wild type, p27$^{-/-}$ and p130$^{+/-}$ p27$^{+/-}$ MEFs (Figures 1,5). But, the absence of both p27 and p130 resulted in cyclin E–Cdk2 kinase activity becoming mitogen-independent (Figure 5b).

Figure 5



Combined deficiency of p27 and p130 results in mitogen-independent cyclin E–Cdk2 activity. (**a**) MEFs of the indicated genotypes were harvested either when proliferating (high serum, Hi) or after serum starvation (low serum, Lo). Whole-cell extracts were immunoblotted using antisera against p130, cyclin E (CycE), p107, Cdk2, p27 and p21. Cyclin E immunoprecipitates were also analyzed for the presence of associated Cdk2 and p21. (**b**) Cyclin E immunoprecipitates from the cell extracts described in (a) were analyzed for associated histone H1 kinase activity.

Regulation of cyclin E–Cdk2 in T lymphocytes

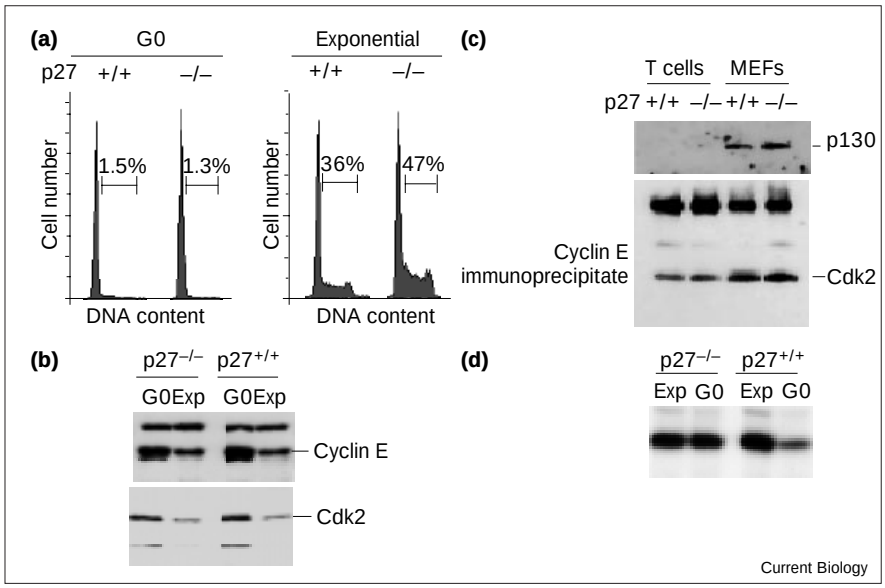
The above results show that p130 can impose mitogen-dependent regulation of the cyclin E–Cdk2 complex in the absence of p27. We would therefore predict that in cell types that normally express very low amounts of p130, mitogenic regulation of cyclin E–Cdk2 may depend exclusively on p27. More than 98% of T lymphocytes purified from the spleens of either wild-type or p27$^{-/-}$ mice are quiescent, and can be stimulated to re-enter the cell cycle by a variety of mitogenic stimuli, such as the lectin concanavalin A (ConA; Figure 6a). In quiescent T lymphocytes, p27 is expressed at high levels, is downregulated by mitogen stimulation, and the inactive cyclin E–Cdk2 complexes in quiescent cells are quantitatively bound to p27 [59,60]. In quiescent T cells p21 is not expressed, but it is induced by mitogens and present at high levels in proliferating cells [60]. Equal amounts of cyclin E and Cdk2 were expressed in proliferating and quiescent p27$^{+/+}$ and p27$^{-/-}$ T cells (Figure 6b). Also, the amount of cyclin E–Cdk2 complexes present in quiescent lymphocytes was equal to that present in quiescent MEFs, and was not affected by p27 status (Figure 6c).

In each of these respects, therefore, T cells and MEFs appear to be similar. An important difference, however, is that quiescent T lymphocytes express much lower amounts of p130 than do MEFs (Figure 6c). This correlates with the observation that cyclin E–Cdk2 complexes are catalytically inactive when prepared from quiescent wild-type T cells, but in p27$^{-/-}$ T cells the cyclin E–Cdk2 kinase is equally active whether the cells are quiescent or proliferating (Figure 6d). Therefore, p27$^{-/-}$ T cells, which naturally express low amounts of p130, have a defect in regulation of the cyclin E–Cdk2 complex similar to that seen in p27$^{-/-}$p130$^{-/-}$ MEFs. We conclude that the level of p130 expression determines how effectively a quiescent cell can compensate for the absence of p27. In p27$^{-/-}$ MEFs, the amount of p130 is just adequate to regulate the normal endogenous amount of cyclin E, but in T lymphocytes the amount of p130 is so low that in the absence of p27, cyclin E–Cdk2 is constitutively active.

Discussion

When normal somatic cells are starved of mitogens, the CKI p27 is induced, both by an increase in its rate of translation and by a decrease in its rate of proteolysis [61–64]. The end result is that in a mitogen-starved, quiescent cell almost every cyclin–Cdk complex is rendered catalytically inactive through its association with a CKI, primarily p27. Moreover, forced ectopic expression of p27 blocks cell proliferation [65–67], and antisense inhibition of p27 expression in mitogen-starved cells delays both the downregulation of CDK activity and exit from the cell cycle [43,44]. On the

Figure 6



Regulation of cyclin E–Cdk2 in wild-type and $p27^{-/-}$ T lymphocytes. (a) Flow cytometric analysis of murine T lymphocytes from wild-type (+/+) and $p27^{-/-}$ mice that were either freshly isolated (quiescent, G0) or stimulated with 10% serum, ConA and phorbol ester (PMA) for 48 h (Exponential). The proportion of cells in S–G2/M is indicated as a percentage. (b) Cyclin E (top) and Cdk2 (bottom) expression was determined by immunoblotting whole-cell extracts from freshly isolated quiescent (G0) and proliferating (Exp) $p27^{-/-}$ and $p27^{+/+}$ T lymphocytes. (c) Western blots for p130 were performed on extracts from freshly isolated quiescent wild-type ($p27^{+/+}$) and knockout ($p27^{-/-}$) murine T lymphocytes and compared to p130 levels in serum-starved wild-type and p27 knockout MEFs (top). Cdk2 western blots were performed on cyclin E immunoprecipitates from quiescent T lymphocytes and serum-starved quiescent MEFs of the indicated genotypes (bottom). (d) Quiescent T lymphocytes (G0) and T lymphocytes that had been stimulated with 10% serum, ConA and PMA for 48 h (Exp) were harvested and whole-cell extracts immunoprecipitated with anti-cyclin E antiserum; histone H1 kinase assays were then performed.

basis of these observations, it was concluded that there is a cause-and- effect relationship among mitogen-dependent CDK activity, p27 expression and cell proliferation.

The use of primary cells from genetically modified mice provides a different and perhaps a more critical test of the role of CKIs in mitogen-dependent CDK regulation. By using wild-type, p21-deficient, p27-deficient, p130-deficient and doubly deficient cells we have confirmed the idea that the CKI p27 is a major regulator of CDK activity in mitogen-starved quiescent cells. This is most clearly evident in T lymphocytes, where the regulation of cyclin E–Cdk2 activity by serum mitogens requires p27. In this regard it is interesting that the thymus is the organ most dramatically enlarged in p27-null mice. In embryonic murine fibroblasts the situation is more complex, but the data still point to p27 as the major regulator of Cdk2 activity in mitogen-starved normal cells. In serum-starved MEFs p27 is induced to high levels and is quantitatively associated with catalytically inactive cyclin E–Cdk2; but we show that in MEFs other proteins can compensate for the absence of p27.

By studying CDK regulation in the absence of p27, we have discovered a new pathway for mitogen-dependent CDK regulation. This pathway involves the tight binding of p130 to cyclin E–Cdk2 complexes, with consequent inhibition of Cdk2 catalytic activity toward exogenous substrates. We show not only that cyclin E–Cdk2 complexes prepared from serum-starved $p21^{-/-}p27^{-/-}$ cells are inactive and quantitatively bound to p130, but also that p130 can inhibit Cdk2 catalytic activity both *in vivo* and *in vitro*. Most significantly, the cyclin E–Cdk2 complex is constitutively active and fully mitogen-independent in MEFs that lack both p27 and p130.

The mechanism of CDK inhibition by p130 remains to be established. The p130 protein is not simply a competitive inhibitor of CDK activity, because the amino-terminal portion of the p130 protein does not contain a CDK phosphorylation site but is nevertheless a potent CKI *in vitro*. One possibility is that p130 will fit the paradigm of tight binding CKIs, like $p40^{Sic1}$ in *S. cerevisiae*, and the p21/p27 proteins in mammalian cells [46,65,68]. Thus, the conformation of the CDK catalytic site might be altered by p130, much as in the p27–cyclin A–Cdk2 complex [69]. Indeed, p130 has the 'RxL' (Arg–X–Lys) motif, which is present in other cyclin-binding proteins and is required for CDK inhibition by members of the p21/p27 family [15]. It is not clear, however, whether or not this motif is required for p130 to inhibit cyclin E–Cdk2 *in vitro*, because it is absent from the amino-terminal inhibitory fragment of p130. Hence, its mode of inhibition may not mimic the one employed by the p21/p27 proteins.

Compensatory or redundant effects of cell cycle regulators have been seen before, but these have all been among family members. For instance, upregulation of the Rb-family member p107 is seen in p130-null T cells [70]. Mice lacking both p27 and p57 show more severe

proliferative abnormalities in the lens than do mice lacking either CKI alone [55]. It is remarkable that in the experiments described here the cell uses p130, a seemingly unrelated protein, to replace p27. Perhaps the most important question raised by our results, therefore, is whether or not CDK inhibition is a normal function of p130. The fact that p130 is normally upregulated after mitogen starvation suggests that p130 has a physiological function in non-dividing cells. Does this function solely involve inhibition of the function of the transcription factor E2F [57,71], or might p130 also have a normal role in more directly regulating CDKs? In MEFs, the CDK-inhibitory role of p130 is subordinate to that of p27, because when p27 is present very little p130 is bound to Cdk2. We therefore expect that if p130 normally acts as a direct CKI this will occur only in quiescent cells that express limiting amounts of p27, p21 and possibly p57.

Another possibility is that p130 takes over the function of CDK inhibition only in abnormal situations where *bona fide* CDK regulators are absent. Tumor cells represent one setting, albeit a pathological one, in which p130 may play an important role in CDK regulation. Many types of human carcinomas often express low amounts of the p27 protein and high amounts of G1 cyclins, including cyclins D1 and E [72]. Moreover, low p27 and high cyclin expression are each independently predictive of tumor aggression and patient mortality [73–76]. The fact that these tumors often lack functional p53, and hence express very low amounts of p21, places further demands on p130 to act as a CDK regulator. Indeed, p130 may be one of the final safeguards against fully mitogen-independent CDK activity, and measuring its level of expression may provide significant new biological information about these potentially aggressive tumors.

Conclusions

The normal pathway for downregulating Cdk2 activity after mitogen starvation of fibroblasts and T lymphocytes involves inducible binding of the CKI p27 to the cyclin E–Cdk2 complex. Accordingly, in p27-null T cells the activity of the cyclin E–Cdk2 complex is mitogen- independent. Surprisingly, however, in p27-null fibroblasts a new pathway can compensate for the absence of p27 and fully restore mitogen-dependence to Cdk2 activity. This requires the binding of the Rb-related protein p130 to the cyclin E–Cdk2 complex and consequent inhibition of Cdk2 catalytic activity. These observations show that a completely unrelated protein can perform the function normally performed by CKIs. In many human tumors CKIs are not expressed at normal levels, and in this context CDK regulation by p130 may be biologically important.

Materials and methods

MEF generation, stable clones, cell culture and transfections

MEFs were generated from 12 day post coitum mouse embryos of various genotypes. Embryos were harvested, the heads and internal organs were removed and carcasses were minced using a scalpel in a petri dish containing fresh medium. The minced embryo was incubated with trypsin at 37°C for 15 min and then placed in a 6 cm tissue culture dish containing DMEM supplemented with 10% FBS, 5 mM glutamine and penicillin/streptomycin. Cells were incubated for 2 days at 37°C with 5% CO_2 and then counted and re-plated at a density of 3×10^5 cells per 6 cm dish. A portion of the primary cells were frozen at 1×10^6 cells per vial and stored at –150°C while others were maintained on a 3T3 protocol where cells were split every 3 days at a density of 3×10^5 into a 6 cm dish. Vials of primary cells were subsequently thawed once per week, expanded every 3 days and used in experiments. Except for the stable cyclin-E-overexpressing clones, primary cells of less than six passages were used in all experiments. Stable cyclin E clones were established using human cyclin E subcloned into the LXSH vector as previously described [77]. Cell lines were established by infecting MEFs of various genotypes that were less than 20 passages in age and selecting stable cells with 100 μg/ml of hygromycin B. For transient transfection experiments, cells were plated at 1×10^6 cells per 10 cm dish and transfected by the calcium phosphate method. Plasmid DNA (5–10μg) was resuspended in 0.5 ml 122 mM $CaCl_2$ and added dropwise to 0.5 ml 2 × HEPES-buffered saline, mixed once and immediately added dropwise to plates. Cells were incubated overnight in calcium phosphate and subsequently rinsed once in medium and re-fed with fresh medium containing 10% FBS (or 0.1% FBS), incubated an additional 24 h and harvested. For serum-starvation experiments, subconfluent cells plated at a density of 1×10^6 in 10 cm dishes were rinsed once with serum-free medium and then re-fed with medium containing 0.1% FBS and incubated an additional 24–48 h. Flow cytometry was performed as previously described [44].

MEFs were produced with targeted disruptions of both p27 and p130 by intercrossing individual knockout strains [50,78]. The p130-deficient mice were first backcrossed two generations to 129/Sv mice congenic to the p27-deficient animals. The p27/p130 double nullizygous animals were viable but infertile, so double nullizygous MEFs were obtained by intercrossing $p27^{+/-}p130^{-/-}$ animals. Embryos were harvested 12 days post coitum, were genotyped by PCR as described, and fibroblasts were cultivated in DMEM + 10% FBS and penicillin/streptomycin on the 3T3 protocol.

Isolation of T lymphocytes and cell culture

Murine spleens were harvested and a single-cell suspension prepared by passage through nylon mesh (100 μm). Red cells were depleted using a whole blood erythrocyte lysis buffer (R&D Systems, Inc., Minnesota) and the resultant cells resuspended in 5% complete RPMI 1640. Non-lymphocyte cells were eliminated by density gradient centrifugation through a discontinuous Percoll gradient as previously described [60] with the following modifications: gradients were made from 40% to 100% (2 ml each of 40%, 60%, 80%, 90% and 100%) and the cell interfaces between 80% and 100% were removed, washed twice with complete RPMI 1640 and passed through a nylon wool column. Cells washed from the columns were further depleted of accessory cells by complement lysis utilizing an anti-heat-stable-antigen antibody and anti-class II MHC antibody (generously provided M. Allegre, University of Chicago). Cells were > 95% $CD3^+$ by FACS analysis (data not shown) and failed to respond to concanavalin A (Con A). For cell culture, freshly isolated mouse T splenocytes were resuspended in RPMI–HEPES 1640 medium containing 10% fetal bovine serum, 2 mM glutamine and 50 μg/ml gentamycin at a density of 1×10^6 cells/ml. Then 2 μg/ml ConA and 2 ng/ml phorbol myristyl acetate (PMA) were added to the culture media and the cells were incubated for an additional 48 h. Flow cytometry was performed to determine the percentage of cells in different phases of the cell cycle.

Antibodies, plasmids and recombinant proteins

Anti-HA-epitope monoclonal antibodies were obtained from Boehringer Mannheim, anti-human-cyclin E polyclonal antibodies, anti-murine-cyclin E polyclonal antibodies, anti-Cdk2 polyclonal and anti-p130 polyclonal antibodies were obtained from Santa Cruz. Anti-p21 polyclonal antibodies

were obtained from Pharmingen and anti-p130 monoclonal antibodies were obtained from Transduction Laboratories. Anti-p27 polyclonal antibodies were obtained from Santa Cruz and generated in house as previously described [44]. Cyclin E–LXSH and Myc-tagged cyclin E plasmids were generated as previously described [77,79]. HA-tagged Cdk2-AF plasmids, CAK virus comprised of His-tagged cyclin H, Cdk7 and p36^{MAT1}, and cyclin E and HA-tagged Cdk2 virus were generously provided by David Morgan (University of California, San Francisco). The three components of CAK were co-infected into Sf9 insect cells and active recombinant CAK complexes were purified by nickel chromatography as previously described [80]. By protein determination, a 1:1:1 molar ratio of the three CAK components was present in the final purified complexes. CAK activity was verified by using recombinant carboxy-terminal domain of RNA polymerase II protein in kinase assays (generously provided by D. Bentley, Amgen Inc.). Cyclin E and Cdk2 virus were separately infected into Sf9 cells, extracts were made and combined at a 1:1 ratio to form inactive recombinant cyclin E–Cdk2 complexes that were used in the CAK activation experiments. The p130 virus was generated by cloning the entire p130 cDNA into the 1392 transfer plasmid and construction of the baculovirus using the Pharmingen BaculoGold system as described by the manufacturer (Pharmingen). GST and GST–p130 (1–320) bacterial constructs were constructed and purified as previously described [81].

Western blots, immunoprecipitation and depletions

Cells were lysed in a modified RIPA buffer (1% Triton-X 100, 10% Glycerol, 0.1% SDS, 0.5% DOC, 20 mM HEPES at pH 7.4, 100 mM NaCl) containing protease and phosphatase inhibitors (10 μg/ml each of aprotinin, leupeptin and pepstatin, 50 mM NaF, 1 mM sodium vanadate). Protein concentrations were determined by Bradford assay, and 50 μg cell extracts were electrophoresed on 12% polyacrylamide gels. After transferring to PVDF membranes proteins were detected by incubation in appropriate primary antibodies followed by HRP-conjugated protein A or HRP-conjugated protein A/G (Sigma) and ECL detection performed as described by the manufacturer (Amersham).

Immunoprecipitation of lysates from cells was performed as follows: approximately 200 μl cell lysates normalized for protein concentrations and incubated at 4°C for 1 h with appropriated dilutions of antibodies, followed by the addition of 50 μl of a 50% slurry of protein A/G–Sepharose (Pharmacia) suspended in modified RIPA buffer minus the SDS and DOC. After rotating 30 min at 4°C the beads were pelleted and washed four times with modified RIPA buffer (minus SDS and DOC), then quenched in SDS sample loading buffer and separated by SDS–polyacrylamide gel electrophoresis followed by western transfer where indicated. Histone H1 and Rb kinase assays were performed as previously described [81,82]. Western blots for p130 were performed using antibody from Transduction Laboratories while p130 immunoprecipitations were performed using p130 antibody from Santa Cruz. For p27 and p130 depletion analysis, antibody followed by protein A/G sepharose addition was performed twice followed by a third incubation of depleted lysates in protein A/G Sepharose alone to remove any residual antibody. All depleted lysates were normalized for protein concentration following the depletions and prior to cyclin E immunoprecipitation analysis. A p130-competing peptide used as control for 'mock' depleted extracts was purchased from Sigma. Depleted lysates were analyzed by western blots to confirm that either p27 or p130 was completely depleted from the extracts. In all cyclin E immunoprecipitations, excess anti-cyclin E antibody was used and it was confirmed that cyclin E was quantitatively immunoprecipitated from the cell extracts.

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