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Cytoplasmic displacement of cyclin E-cdk2 inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in anchorage-independent cells

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Loss of attachment to an extracellular matrix substrate arrests the growth of untransformed cells in the G1 phase. This anchorage-dependent cell cycle arrest is linked to increased expression of the $p21^{Cip1}$ (p21) and $p27^{Kip1}$ (p27) cyclin-dependent kinase inhibitors. The result is a loss of cdk2-associated kinase activity, especially that of cyclin E-cdk2. The levels of p21 and p27 are also upregulated in unattached transformed cells, but cyclin E-cdk2 activity remains high, and the cells are able to grow in an anchorage-independent manner. Increased expression of cyclin E and cdk2 appears to be partially responsible for the maintenance of cyclin E-cdk2 activity in transformed cells. To explore further the regulation of cyclin E-cdk2 in transformed cells, we have analysed the subcellular distribution of cyclin-cdk complexes and their inhibitors in normal human fibroblasts, their transformed counterparts, and in various human tumor cell lines. In substrate-attached normal fibroblasts, cyclin E and cdk2 were exclusively in the nuclear fraction, associated with one another. When normal fibroblasts were detached and held in suspension, cyclin E-cdk2 complexes remained nuclear, but were now found associated with the p21 and p27 cdk inhibitors and lacked histone H1 phosphorylating activity. In contrast, the transformed fibroblasts and tumor cells, which are anchorage-independent, had more than half of their cyclin E, cdk2, p21 and p27 in the cytoplasmic fraction, both in attached and suspended cultures. The cytoplasmic p21 and p27 were bound to cyclin E-cdk2, as well as to complexes containing cyclin A and cyclin D. The nuclear cyclin E-cdk2 complexes from the transformed cells grown in suspension contained only low levels of p21 and p27 and had histone H1 kinase activity. Thus, at least three mechanisms contribute to keeping cyclin E-cdk2 complexes active in suspended anchorage-independent cells: cyclin E and cdk2 are upregulated, as reported previously, cdk inhibitors are sequestered away from the nucleus by cytoplasmic cyclin-cdk complexes, and the binding of the inhibitors to nuclear cyclin E-cdk2 complexes is impaired.

Keywords: anchorage; p21Cip1; p27Kip1; cyclin E; cdk2; cell adhesion; cell cycle

Introduction

Most normal cells require attachment to a substratum to survive and to grow. This phenomenon is referred to as anchorage dependence, and it is mediated by attachment of cells to extracellular matrix proteins through integrins (Ruoslahti and Reed, 1995). Fibroblasts detached from extracellular matrix stop growing, whereas epithelial and endothelial cells undergo apoptosis (Meredith *et al*., 1993; Frisch and Francis, 1994; Re *et al*., 1994). Anchorage dependence is likely to be important for the maintenance of tissue architecture; it prevents cells from detaching and moving into inappropriate places in tissues.

Detachment of cells from matrix results in a loss of integrin signaling, that results in changes in cell cycle regulation (Koyama *et al*., 1996; Wary *et al*., 1996; Assoian and Zhu, 1996; for review see Assoian, 1997). In human fibroblasts, detachment causes upregulation of the cell cycle inhibitors p21 and p27 and the inhibitors arrest the cell cycle at the G1/S transition by inactivating the cyclin E-cdk2 complex (Fang *et al*., 1996). An increase in the levels of either or both of the inhibitors, accompanied by inactivation of cyclin D-cdk4 or cyclin E/A-cdk2 complexes, has also been observed in other cell types such as NRK, NIH3T3, and Rat1 fibroblasts (Zhu *et al*., 1996; Schulze *et al*., 1996). Similar cell cycle inhibition is also seen in smooth muscle cells growth arrested upon loss of anchorage, or as a result of growing on an inhibitory extracellular matrix (Koyama *et al*., 1996), or disruption of the actin cytoskeleton in normal human fibroblasts (Böhmer *et al*., 1996). Contact inhibition of growth in cells that have grown confluent and attach to one another is also mediated by p27 (Polyak *et al*., 1994; Hengst and Reed, 1996; St Croix *et al*., 1996; Coats *et al*., 1996). Thus, regulating cdk inhibitors appears to be a general pathway used by cells to respond to changes in attachment (for review see Ruoslahti and Öbrink, 1996).

Malignant cells are generally independent of anchorage and this is thought to be of fundamental importance for invasiveness and metastasis (Folkman, 1978). Tumor cells circumvent the anchorage requirement of normal cells by bypassing the signaling pathways triggered by matrix attachment. For example, Src-transformation results in constitutive phosphorylation (activation) of a number of signaling proteins that are normally activated upon integrin-mediated cell attachment (Frisch and Francis, 1994; Richardson and Parsons, 1996) and v-Abl transformation results in enhanced activation of cyclin E-cdk2 complexes and in increased c-*myc* transcription (Zou *et al*., 1997). Malignantly transformed cells also show abnormalities in the pathway from cell attachment to regulation of the cyclin/cdk complexes. Thus, for example, transformed fibroblasts have active cyclin E-cdk2 complexes regardless of whether they are attached and despite the elevation in cdk inhibitor expression that occurs upon detachment from matrix (Fang *et al*.,

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1996). One reason for the lack of proper cyclin E-cdk2 regulation in these cells appears to be an elevated expression of the cyclin E and cdk2 proteins (Fang *et al*., 1996).

We show here that various types of malignantly transformed cells maintain anchorage-independent cyclin E-cdk2 activity by excluding the majority of p21 and p27 from the nucleus, and by compromising the association of nuclearly localized inhibitors with cyclin E-cdk2 complexes.

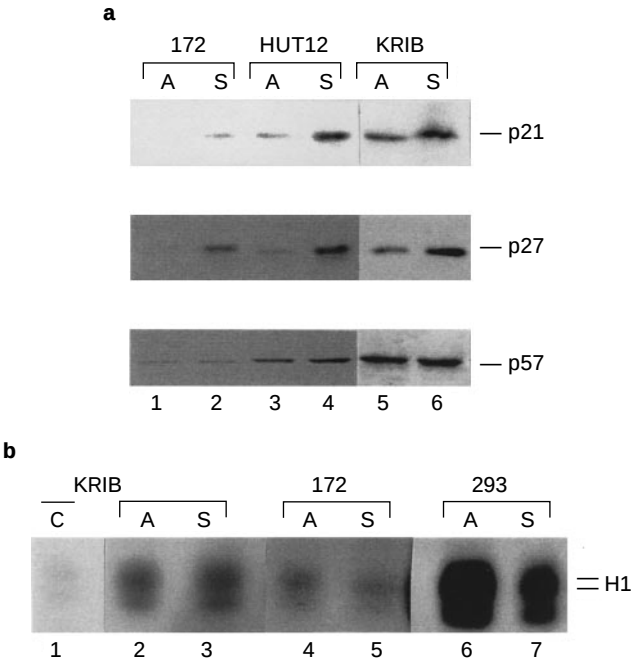

Figure 1 Expression of cdk inhibitors and cyclin E-cdk2 activity in anchorage-independent cell lines. (**a**) Expression of cyclin E-cdk2 inhibitors p21, p27 and p57 in cells cultured in suspension. Equal amounts of attached and suspended cell lysates from HUT12, 172 and KRIB cells were separated in a 4–20% gel and probed for the expression of the inhibitors by immunoblotting. (**b**) *In vitro* histone H1-kinase assay on cyclin E-cdk2. Equal amounts of attached and suspended cell lysates from 172, KRIB and 293 cells were immunoprecipitated with an anti-cyclin E antibody and kinase activity in the precipitate was measured with histone H1 as substrate. Slightly reduced cyclin E-cdk2 activity in detached 293 cells (lane 7) could be explained by cell aggregation in suspension culture, as has been reported for other cell lines (St. Croix *et al*., 1996). C is normal rabbit serum control

Results

Expression of p21 and p27 cyclin-cdk inhibitors in suspended anchorage-independent cells

We have previously shown that p21 and p27 expression is elevated in the transformed cell line HUT12 when they are cultured in suspension (Fang *et al*., 1996). To determine whether this happens in other anchorage-independent cells, we analysed p21 and p27 expression levels in several anchorage-independent cell lines. The results are shown in Figure 1 and summarized in Table 1. Complex patterns of inhibitor expression were found: Some of the cells expressed less p21 or both p21 and p27 than anchorage-dependent fibroblasts, but nevertheless responded to loss of adhesion with increased expression of the inhibitors; up to fourfold increases in p21 and p27 were seen (e.g. HUT12 and 172 in Figure 1a). p57 expression did not change in suspended cells. Despite the elevated p21 and p27 levels, the cyclin E-cdk2 complexes remained active in all these transformed cell lines in suspension culture (Figure 1b and Table 1).

Cytoplasmic displacement of p21 and p27

To determine the reasons for the retention of cyclin E-cdk2 activity in the suspended anchorage-independent cell lines, we studied the subcellular localization of p21 and p27 by cell fractionation. Whereas both inhibitors were exclusively nuclear in suspended normal fibroblasts (Figure 2a, top lanes 1–4), a considerable fraction, sometimes a majority of both proteins, was in the cytoplasmic fraction in suspended anchorage-independent cells (Figure 2a).

The completeness of the subcellular fractionation was evaluated by studying the distribution of known nuclear and cytoplasmic marker proteins in one of the cell lines, HUT12. α-tubulin, a commonly used cytoplasmic marker, was found exclusively in the cytoplasmic fraction, whereas histone H3 was exclusively nuclear (Figure 2b). Two nuclear proteins, the faster migrating and active form of cdk7 and cyclin H were found in the nuclear fraction of the HUT12 cells (Figure 2a), as well as of normal fibroblasts (Table 2). Moreover, a slower migrating form of p27 was present in the cytoplasmic, but not the nuclear fraction of

Table 1 Summary of cdk inhibitor analyses in various cell lines

Cell line	*DNA replication*	*cyclin E-cdk2 activity*	*cdk2 thr-160*[a]	*Inhibitors (b)*			*Inhibitors complexed with cyclin E-cdk2 (d)*		
				p21	*p27*	*p57*	*p21*	*p27*	*p57*
KD	–	–	–	4[d]	4	1–2	4	4	–
FS	nd[c]	–	–	4	4	1–2	4	4	–
IMR-90	–	–	–	4	4	nd	nd	nd	nd
EMFI	nd	nd	–	4	4	nd	nd	nd	nd
Rat1	nd	–	+	nd	4	nd	nd	2	nd
172	nd	+	+	4	4	–	4	4	nd
HUT12	+	+	+	4	4	–	4	4	nd
KRIB	+	+	+	4	4	–	2–4	4	nd
MG-63	nd	+	+	4	4	–	2–4	4	nd
SKUT1	nd	+	+	4	4	nd	nd	nd	nd
293	nd	+	+	1	4	1	1	1	nd

[a]active form of cdk2. [b]compared to attached cells. [c]not done. [d]4 = highest level of expression;

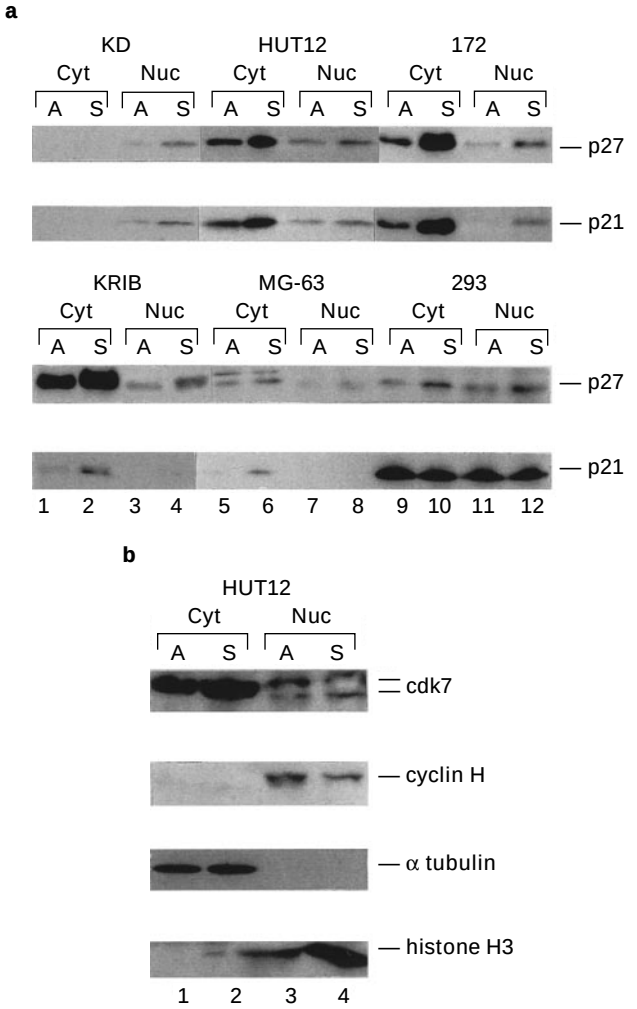

Figure 2 Subcellular localization of cdk inhibitors. (**a**) p21 and p27 in cytoplasmic and nuclear fractions of cells. Equal amounts of the fractions (30–50 μg) from KD, HUT12, 172, KRIB, MG-63 and 293 cells were analysed for p21 and p27 expression by immunoblotting. The MG-63 and 293 blots were equally exposed, showing a much reduced p21 expression in the MG-63 cells. For the KD cells, cdk2-associated p21 and p27 is shown after immunoprecipitating cdk2 and probing the immunocomplexes for the inhibitors. The cytoplasmic lysates were devoid of p21 and p27, confirming exclusively nuclear localization (lanes 3 and 4) of these inhibitors in normal fibroblasts (data not shown). (**b**) Controls for the subcellular fractionation. The distribution of the nuclear markers cyclin H, the fast-migrating, active form of cdk7 and histone H3, and the cytoplasmic marker α-tubulin was analysed by immunoblotting. Equal amounts (50 μg) of cytoplasmic and nuclear fractions from attached and suspended HUT12 cells were tested with the appropriate antibodies

MG-63 cells (Figure 2a). These results clearly validate the subcellular fractionation procedure.

To confirm the subcellular localizations of p21 and p27 inferred from the cell fractionation, we carried out immunofluorescence staining of fixed cells (Figure 3). The staining for p21 and p27 was entirely nuclear in the MRC-5 normal fibroblasts, whereas substantial cytoplasmic staining was seen in the anchorage-independent cell lines HUT12, KRIB and 172 (data not shown). As expected, histone H3 was exclusively nuclear in HUT12 cells, whereas α-tubulin was cytoplasmic.

Table 2 Summary of subcellular cyclin/cdk/cdk inhibitor distribution in normal and transformed cells

	Normal fibroblasts[a]		*Transformed cell lines*[b]	
	C	*N*	*C*	*N*
p57	–	+	+[d]	+
p27	–	+	+	+
p21	–	+	+	+
cdk2	–	+	+	+
cyclin E	–	+	+	+
27/E-2	–	+	+	+
21/E-2	–	+	+	+
cyclin A	–	+	+	+
cyclin D	–	+	+	+
cdk4	nd	nd	+	+[e]
cyclin H	–	+	+[c]	+[c]
cdk7	–	+	+[c]	+[c]
PCNA	nd	nd	+	+

[a]KD and FS cells. [b]HUT12, 172, KRIB, MG63 and 293 cells. [c]Molecular weights of cytoplasmic and nuclear molecules were different. [d]Not cytoplasmic in 172 cells. [e]Not nuclear in MG-63 cells

Cyclin E and cdk2 in anchorage-independent cells

Cyclin E and cdk2 levels are higher in anchorage-independent HUT12 cells than in anchorage-dependent fibroblasts (Fang *et al*., 1996). An increase in cyclin E-cdk2 complex levels and potentially compensate for the increase in p21 and p27 that takes place in suspension cultures, allowing HUT12 cell growth to be anchorage-independent. To determine whether the increase in cyclin E-cdk2 complexes plays a role in the altered distribution of p21 and p27 in transformed cells, we compared the subcellular localization of cyclin E and cdk2 in HUT12 cells and normal fibroblasts. Immunoblotting of nuclear and cytoplasmic fractions showed that cyclin E and cdk2 were up to 40% more abundant in the cytoplasmic than nuclear fraction of HUT12 cells in late G1 (Figure 4a, lanes 1–4). Similar results were obtained with the 172, KRIB, MG-63 and 293 cells (Table 2). In contrast, both proteins were exclusively nuclear in normal KD fibroblasts (Figure 4a, lanes 5–8).

Even though a considerable fraction of the cyclin E and cdk2 protein of the HUT12 cells was in the cytoplasmic fraction, the nuclear fraction of these cells still contained about sevenfold more cyclin E and cdk2 than that of the normal fibroblasts (data not shown). Immunoprecipitation with anti-cdk2 followed by blotting with anti-cyclin E revealed the presence of cyclin E-cdk2 complexes in the cytoplasmic fraction of the HUT12 cells (Figure 4b). However, not all the cytoplasmic cdk2 was cyclin-associated, because a significant fraction remained in the cyclin E-depleted supernatant (data not shown). The presence of non-cyclin associated cdk2 was also suggested by the observation that less than half of the cytoplasmic cdk2 was in the Thr160-phosphorylated form, which is characteristic of cyclin-associated cdk2.

The localization of cyclin E in both the nucleus and cytoplasm of the anchorage-independent cells was confirmed by immunostaining with anti-cyclin E antibodies. Nuclear and cytoplasmic staining was obtained with polyclonal and monoclonal anti-cyclin E antibodies in both the HUT12 and KRIB cells. The HUT12 staining with the polyclonal antibodies is shown in Figure 5. In agreement with the cell fractionation experiments, the MRC-5 normal fibroblasts showed exclusively nuclear cyclin E staining.

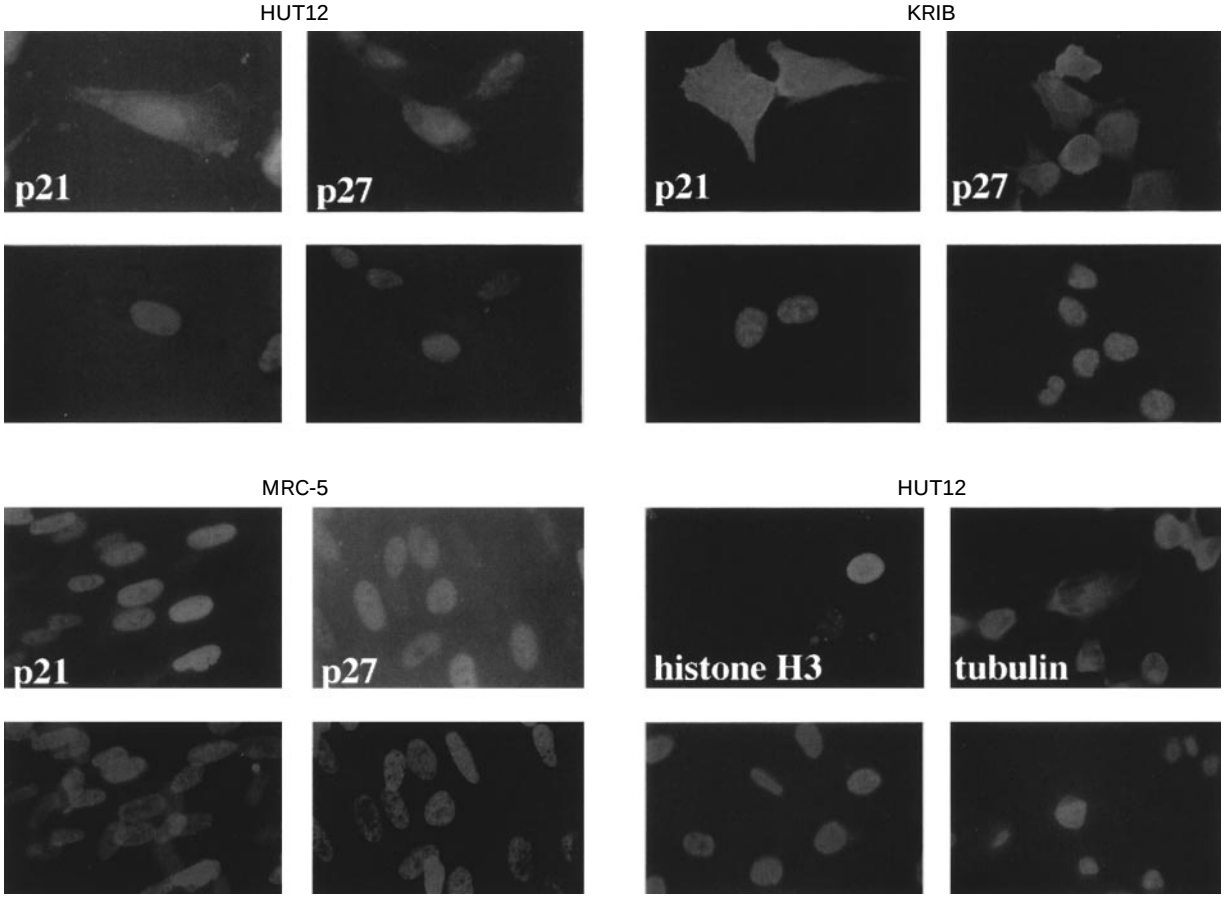

Figure 3 Localization of p21 and p27 by immunofluorescence staining. Asynchronously growing HUT12 cells and contact-inhibited normal MRC-5 fibroblasts were fixed and stained for expression of the cdk inhibitors (upper panels for each cell line). The localization of the nuclear marker histone H3 and the cytoplasmic marker α-tubulin in the HUT12 cells is also shown (lower right). Histone 3 is seen in two cells. The nuclei in each microscopic field were visualized with Hoechst-33258 (unlabeled panels)

Association of nuclear and cytoplasmic cyclin E-cdk2 with p21 and p27

We next studied the association of p21 and p27 with the cyclin E-cdk2 complexes in the subcellular compartments. Cyclin E and cdk2 were immunoprecipitated from nuclear and cytoplasmic fractions of five anchorage independent cell lines, grown as either attached or suspended cultures, and assayed for associated inhibitors by immunoblotting. In each case, the immunoprecipitated protein was recovered in the precipitate, with only occasional trace amounts in the supernatant (data not shown). The results of the inhibitor analysis are shown for three representative cell lines in Figure 6a. All of the cell lines had p21 and p27 associated with cyclin E-cdk2 complexes in the cytoplasmic fraction, more so in suspension than in attached cultures. In general, the nuclear cyclin E-cdk2 complexes from the anchorage-independent cells reproducibly contained very little or no associated p21 or p27 (Figure 6), even though some of the lines had significant nuclear levels of these inhibitors (Figure 2a). KRIB (Figure 6) and MG-63 (data not shown) cells showed no detectable inhibitors associated with their nuclear cyclin E-cdk2 complexes, even when twice as much KRIB nuclear extract was used. More p21 and p27 was bound to cdk2 than to cyclin E in 293 (Figure 6) and 172 (data not shown) cells, indicating that cdk2-complexes other than cyclin E-cdk2 had bound some of the inhibitors. These results were reproducible in several independent experiments. Moreover, the nuclear cyclin E-cdk-2 complexes from attached and suspended KRIB cells were active in phosphorylating histone H1 (Figure 6b).

Cytoplasmic p21 and p27 are partially associated with cyclin A and D

We also looked for possible association of p21 and p27 with cyclin A, cyclin D, and cdk4. Each of these proteins was present in the cytoplasm of the various anchorage-independent cells, as determined by immunoblotting (Figure 4a and Table 2) and immunofluorescence (Figure 5). A detailed analysis was performed in the HUT12 cells (Figure 7). We prepared immunoprecipitates from cytoplasmic and nuclear fractions of HUT12 cells with antibodies against p21 and p27 and immunoblotted them with anti-cyclin A, anti cyclin D and anti-cdk4 (Figure 7a). Alternatively, we immunoblotted cyclin and cdk4 immunoprecipitates with anti-p21 and anti-p27 (Figure 7b). In each case, we analysed primary and secondary immunoprecipitates as well as the remaining supernatants to account for all of the protein that was being analysed.

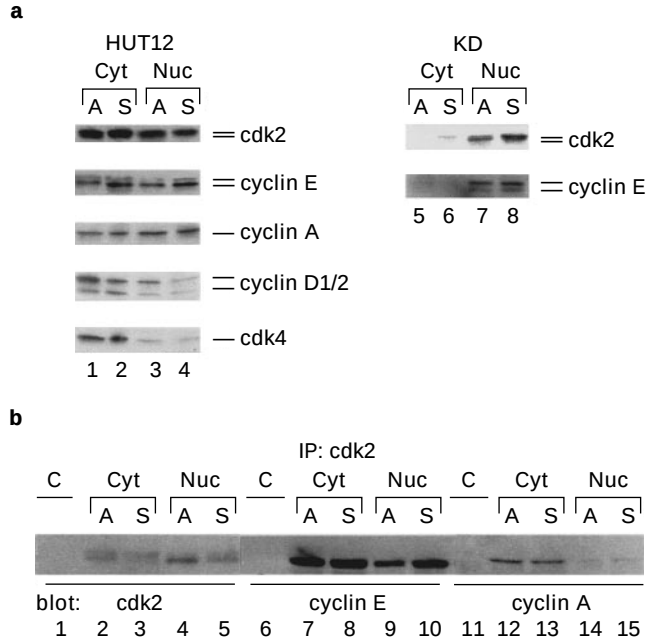

Figure 4 Subcellular localization and complexing of cyclins and cdks. (**a**) Cyclins and cdks in cytoplasmic and nuclear fractions. The fractions (50 μg) from attached and suspended HUT12 cells (lanes 1–4) and KD fibroblasts (lanes 5–8) were analysed for the presence of cyclins D1/2, E and A, cdk2 and cdk4 by immunoblotting. (**b**) Complexing of cytoplasmic and nuclear cyclins E and A with cdk2. Cytoplasmic and nuclear fractions (50 μg) from attached and suspended HUT12 cells were immunoprecipitated with an antibody against cdk2, and cyclins E and A were detected in the precipitates by immunoblotting with specific antibodies. The lanes marked with C are controls with the anti-cdk2 antibody and protein A Sepharose, but no cell fraction. The identity of cdk2, cyclin E and A was confirmed by comparing the mobility to that of the same proteins in whole cell lysates (data not shown)

The results showed that a fraction of p21 and p27 is associated with cyclins A and D in the HUT12 cytoplasm (Figure 7a). The amounts were comparable to those associated with cyclin E. Cdk4 immunoprecipitates also contained some of the inhibitors, presumably because of the association of cdk4 with cyclin D (La Baer *et al*., 1997). Most of the nuclear cyclin D was bound to the inhibitors, because it was not detected in the supernatants depleted of p21 and p27 (Figure 7a, lanes 7 and 15). Some cytoplasmic, but essentially no nuclear cdk4, was bound to the inhibitors (Figure 7a, lanes 5 and 13; Figure 7b, lane 5).

Whereas the cytoplasmic cyclin E and A complexes contained abundant p21 and p27 (Figure 7a, lanes 2 and 10; Figure 7b, lane 2), only a minor fraction of cyclins E and A was found in the inhibitor immunoprecipitates from the nucleus (Figure 7a, lanes 5 and 13, Figure 7b, lane 5). Instead the cyclins were found in the inhibitor-depleted supernatants (Figure 7a, lane 7 and 15).

p21 can also interact with PCNA, and this interaction is believed to contribute to a p21-mediated cell cycle arrest (reviewed in Kelman, 1997). We found PCNA to be overexpressed in HUT12 cells compared to normal fibroblasts (data not shown). In addition to confirming its expected localization in the nucleus, we also found PCNA in the cytoplasmic fractions of HUT12 (Figure 7a) and of the other anchorage-independent cells (Table 2). Some of the cytoplasmic PCNA was bound to p21 (Figure 7a, lane 10). In contrast, nuclear PCNA was essentially free of p21 (Figure 7a, lane 13).

One of the anchorage-independent cell lines we studied (293), expresses adenovirus (Ad) E1A. E1A has been shown to bind p27 (and to p21; R Poon and M Harter, personal communication) and can prevent the association of p27 with cyclin-cdk2 complexes when overexpressed in transfected cells (Mal *et al*., 1996). Immunoblotting of p27 immunoprecipitates for E1A showed that E1A is associated with p27 in both the nuclear and the cytoplasmic fractions of the 293 cells, with more p27-associated E1A in the cytoplasmic fraction (Figure 8). Since p27 was also found associated with cyclin E-cdk2 in 293 cells (Figure 6), we deduce that a fraction of the p27 population is not bound to E1A, but E1A may nonetheless contribute to the neutralization of the cyclin inhibitors in these cells.

Discussion

We show here that several transformed and tumor cell lines, unlike normal fibroblasts, have a major portion of their cyclin E and cdk2 located in the cytoplasm. The cytoplasmic cyclin E-cdk2 and other cytoplasmic cyclin-cdk complexes are bound to cdk inhibitors p21 and p27, providing one mechanism for maintaining anchorage-independent activity of nuclear cyclin E-cdk2 complexes in tumor cells.

Changes in the expression and activities of cyclin-cdk complexes have been implicated in anchorage independence, one of the fundamental properties of transformed and tumor cells. Some anchorage-dependent cell lines downregulate their cyclin D expression when they lose anchorage to a substrate (Zhu *et al*., 1996), while in normal fibroblasts loss of adhesion results in inactivation of the cyclin E-cdk2 complex by cdk inhibitors (Fang *et al*., 1996). Transformed and tumorigenic cell lines are independent of anchorage, and this trait correlates with sustained activity of cyclin E-cdk2 in cells taken into suspension (Fang *et al*., 1996; this study). Like normal cells, anchorage-independent transformed cells upregulate the p21 and p27 cdk inhibitors upon loss of anchorage (Fang *et al*., 1996), and this would be expected to cause cell cycle arrest. Increased expression of cyclin E and cdk2 (Keyomarsi *et al*., 1994, 1995; Dutta *et al*., 1995; Kitahara *et al*., 1995; Miyajima *et al*., 1996; Weinstat-Saslow *et al*., 1995) appears to be one of the mechanisms transformed cells use to neutralize the cdk inhibitors.

The neutralization of the cdk inhibitors by cyclin-cdk complexes in anchorage-independent cells appears to take place in the cytoplasm. Cytoplasmic expression of cyclin E, cdk2 and other cyclins correlated with the anchorage-independent, transformed phenotype in our study. We detected no cytoplasmic cyclins or cdk2 in two anchorage-dependent cell lines, whereas five transformed and tumorigenic cell lines all expressed a major portion of these proteins in the cytoplasm. Differences in the fractionation of proteins in normal and transformed cells into nuclear and cytoplasmic fractions cannot account for the cyclin-cdk distribution

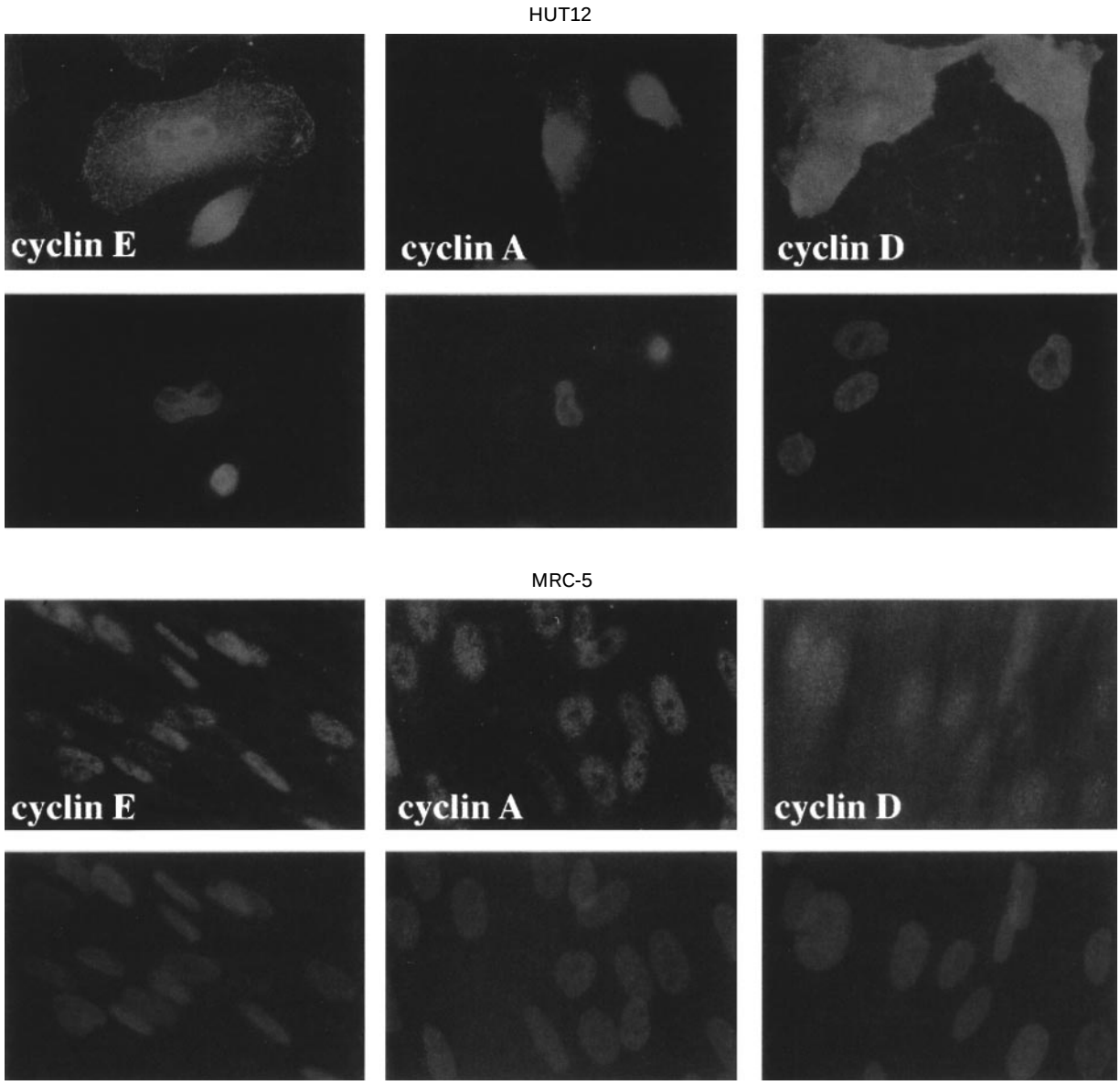

Figure 5 Localization of cyclins by immunofluorescence staining. HUT12 cells and normal MRC-5 fibroblasts were fixed and stained for cyclins as indicated. The nuclei in each field were visualized with Hoechst-33258 (unlabeled panels)

difference, because nuclear and cytoplasmic markers distributed in the expected fractions in each cell type. Moreover, immunostaining of the cyclin and cdk proteins *in situ* confirmed the cytoplasmic localization in transformed cells. Thus, our results appear to be an accurate reflection of the presence of substantial quantities of cyclin and cdk proteins in the cytoplasm of transformed and tumor cell lines. To our knowledge this is the first report on cytoplasmic localization of a major fraction of cyclins and cdk's.

Cyclins E and A lack a nuclear localization signal (NLS) that would allow their entry into the nucleus on their own (Robbins *et al*., 1991) but association with a cdk can effect nuclear translocation (Maridor *et al*., 1993). We find that, despite having formed a complex with cdk2, cyclin E and A remain partially in the cytoplasm. The mechanism whereby the cyclin-cdk complexes are retained in the cytoplasm remains to be elucidated. However, it is noteworthy that cyclin E is toxic when overexpressed in yeast (Sewing *et al*., 1994) and uncouples DNA replication from the cell cycle in fibroblasts (Mumberg *et al*., 1996). Thus, the overexpression of cyclin E that is strongly correlated with malignant transformation (this study; Keyomarsi *et al*., 1994; Dutta *et al*., 1995; Weinstat-Saslow *et al*., 1995; Dou *et al*., 1996), may be possible only when most of the overexpressed cyclin E remains cytoplasmic, as it does in the transformed and tumor cells we have studied.

Both, p21 and p27 have an NLS that could facilitate nuclear entry of associated cyclin E-cdk2 complexes as was recently demonstrated for cyclin D-cdk4 complexes (LaBaer *et al*., 1997). Such transfer is obviously impaired in our tumor cell lines, because we detect inhibitor-containing cyclin E-cdk2 complexes in the cytoplasm. Cyclin E-cdk2(-inhibitor) complexes in transformed cells could be associated with a protein that causes cytoplasmic retention was recently shown for JIP-1 associated JNK (Dickens *et al*., 1997). A cytoplasmic retention signal can be dominant over the

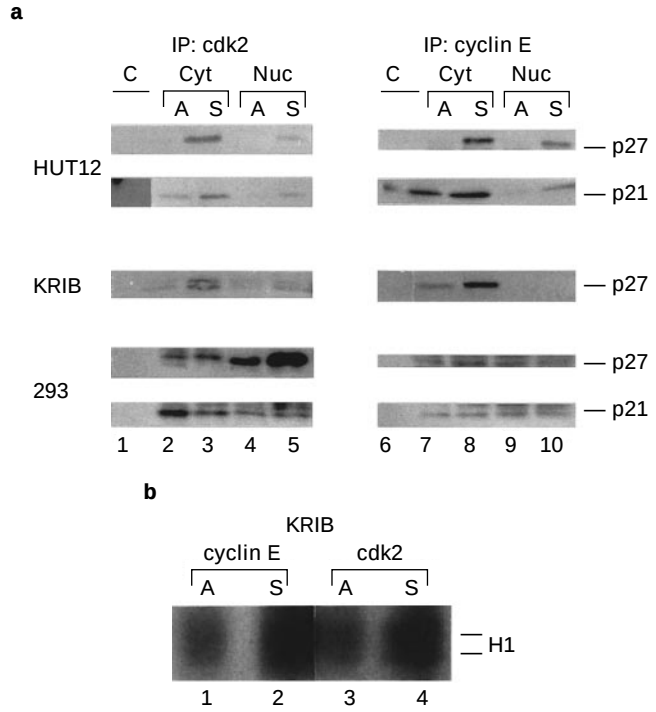

Figure 6 Subcellular localization of the inhibitors in cdk2 and cyclin E immunocomplexes and kinase activity of the nuclear complexes. (**a**) Cdk2 (left panels) or cyclin E-immunoprecipitates (right panels) of 50 μg of cytoplasmic and nuclear lysates from HUT12, 293 cells and 100 μg from KRIB cells were immunoblotted for p21 and p27 after separation in 4–20% PAGE. (**b**) *In vitro* kinase assay on cyclin E and cdk2 immunoprecipitates from nuclear lysates on histone H1 substrate

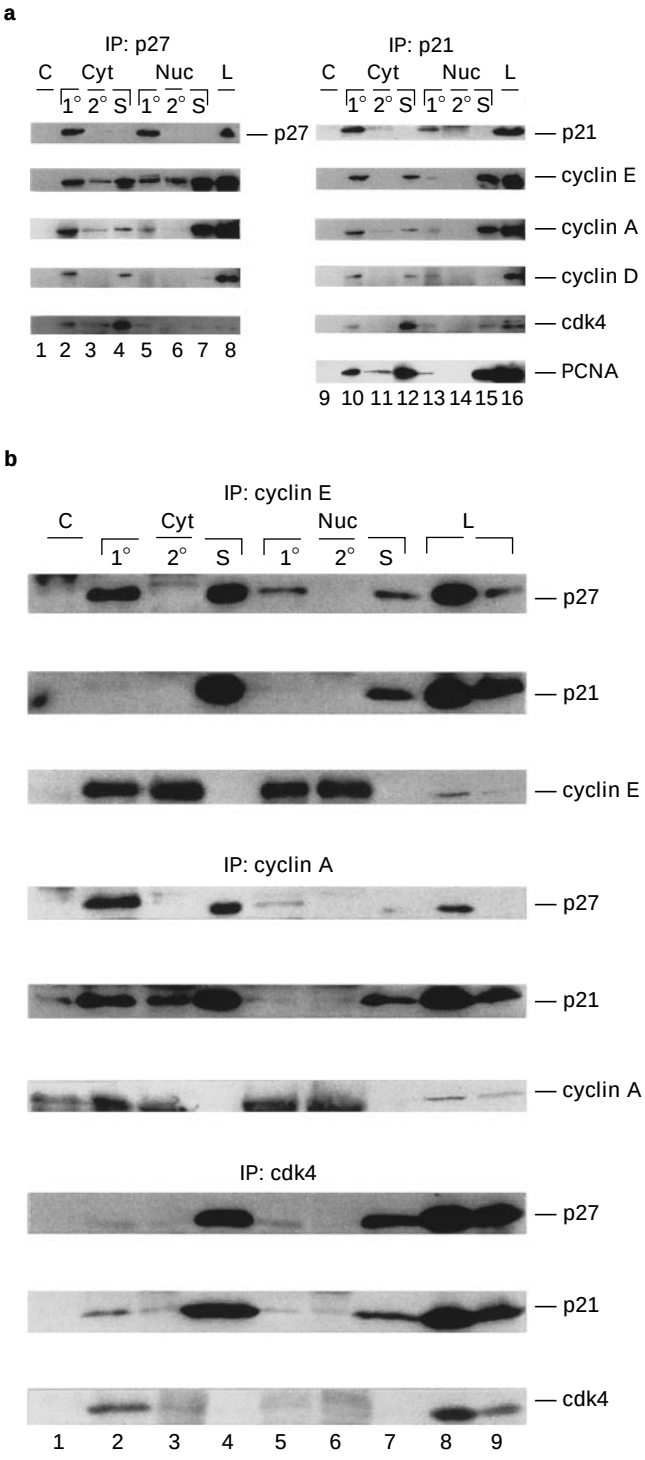

Figure 7 Association of cyclins, cdk4 and PCNA with cdk inhibitors in fractionated HUT12 cell lysates. (**a**) p21 and p27 immunoprecipitates. Cytoplasmic (lanes 2–4 and 10–12) and nuclear fractions or total cell (L) lysates (50 μg) of suspended HUT12 cells (5–7 and 13–15) were immunoprecipitated twice (1° and 2°) with anti-p27 (left panel) or anti-p21 (right panel) antibodies. The absence of the inhibitor in the supernatants (lanes 4, 7, 12 and 15) shows that the immunoprecipitation was complete. The immunoprecipitates and supernatants were immunoblotted for cyclins E, A, D, cdk4 and PCNA after separation on 4–20% PAGE. (**b**) Cdk inhibitors in cyclin E, cyclin A and cdk4 immunoprecipitates. The immunoprecipitates and their supernatants from cytoplasmic, nuclear or total cell (L) lysates of suspended HUT12 cells were immunoblotted for p21 and p27 after separation on 4–20% PAGE. The immunoprecipitation was shown to be complete by probing the supernatants with the appropriate antibody (bottom row in each panel)

NLS as is the case with cyclin B-cdc2 complexes (Pines and Hunter, 1994).

The binding to the cytoplasmic cyclin-cdk complexes is likely to sequester the inhibitors away from the critical nuclear cyclin-cdk complexes. Thus, we propose that the cytoplasmic cyclins may neutralize the inhibitors in transformed and tumorigenic cells, leaving the nuclear cyclin-cdk complexes active and capable of promoting rapid and inappropriate cell cycling. Cytoplasmic cyclin D-cdk4/6-p15 inhibitor complexes have been described in a different role; in Mv1Lu cells that inducibly express p15 cytoplasmic p15 prevents cyclin D-cdk4/6 from binding p27, thus leaving p27 free to enter the nucleus and block cell cycle progression by inactivating other cyclin-cdk complexes (Reynisdóttir and Massagué, 1997).

The overexpressed and cytoplasmic cyclin-cdk complexes may not be the only factor securing cyclin E-cdk2 activity in unattached transformed cells. We find that although some fraction of p21 and p27 is nuclear in such cells, it is mostly not associated with cyclin E-cdk2. Other binders of the cdk inhibitors such as the adenovirus E1A protein (Mal *et al*., 1996), present in one of our cell lines, human papillomavirus E7 protein (Zerfass-Thome *et al*., 1996) and possibly cellular counterparts of such viral proteins may contribute to the impairment of cdk inhibitor function. c-Myc-induced sequestration of p27 in a form that is unable to bind and inhibit cyclin E-cdk2 (Vlach *et al*., 1996) is another possible mechanism for neutralizing the inhibitors. That multiple mechanisms

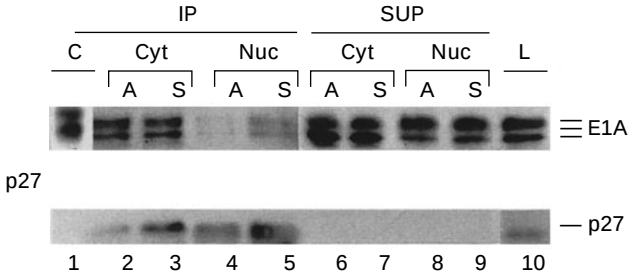

Figure 8 Association of p27 with adenovirus E1A in 293 cells. Cytoplasmic and nuclear fractions (50 μg) from 293 cells were immunoprecipitated with anti-p27 (IP), separated on a 4–20% PAGE together with the p27-depleted lysates (SUP) and immunoblotted for E1A (upper panel), and for p27 (lower panel) to confirm complete immunoprecipitation C is normal serum control; L is immunoprecipitate from total cell

exist to maintain anchorage-independent activity of cyclin E-cdk2 in transformed and tumorigenic cells is likely to reflect the importance of cyclin E for anchorage independent cell growth.

Materials and methods

Cell culture and cell fractionation

Normal human fibroblasts KD (Kakunaga, 1978), FS (foreskin), IMR-90, and MRC-5, embryonic mouse fibroblasts (EMFI), and rat fibroblasts (Rat-1) were cultured in 14 cm dishes in Dulbecco's minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Cells were used at passage 20 or below except EMFI, which were used at or below passage 5. HUT12 (a chemically transformed KD cell line; Kakunaga, 1978), 172 MDAH (a spontaneously immortalized fibroblastic cell line from a Li-Fraumeni patient; Medcalf *et al*., 1996), KRIB (a Ki-Ras overexpressing osteosarcoma cell line; Samid and Mandler, 1989), MG-63 and SK-UT-1 (human osteosarcoma cell lines), and 293 cells (Ad5 E1A-expressing human embryonic kidney cell line) were cultured as described above for the normal fibroblasts. All cell lines were from the American Type Culture Collection if not indicated otherwise.

Fibroblasts and HUT12 cells were cultured in monolayer or suspension cultures and were synchronized by serum starvation (Fang *et al*., 1996). Serum-starved cells were scraped from the tissue culture dishes, suspended either in 10% FCS/α-MEM before reattachment or were kept in 1% Methocel (Rheinwald and Green, 1974) in 10% FCS/α-MEM for the suspended cultures for 7 h (G1) and 14–18 h (G1/S). The suspension culture was then sealed in a 50 ml Corning tube and incubated in a rolling incubator at 37°C. Cells were lysed either in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM NaF, 0.5 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin, 0.1 U/ml aprotinin) or in 0.1% NP-40 buffer (50 mM Tris-HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 15 μg/ml benzamidine, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, 4 μg/ml pepstatin, 0.1 U/ml aprotinin), or were subjected to subcellular fractionation according to a modified protocol from Dignam *et al*. (1983). In the fractionation, the cells were left to swell on ice for 30–45 min in hypotonic buffer A (1.5 mM $MgCl_2$, 10 mM HEPES pH 7.9, 10 mM KCl, 0.5 mM DTT, 10 μg/ml leupeptin, 4 μg/ml pepstatin, 0.1 U/ml aprotinin, 15 μg/ml benzamidine, 10 μg/ml trypsin inhibitor) before the cell membranes were disrupted by shearing the cells three times for 10 s in a blender (OMNI-1000, Waterbury, CT). Disruption of the cell membrane was confirmed under the microscope. The cytoplasm was separated from the nuclear fraction by centrifugation for 10 min at 10 000 r.p.m. in the cold and was frozen at liquid nitrogen temperature. Proteins from the nuclear pellet were recovered either by three rounds of extraction with buffer C (0.5% NP-40, 10 mM Na-phosphate, pH 7.4, 120 mM NaCl, 0.1 mM PMSF, 10 μg/ml leupeptin, 4 μg/ml pepstatin, 0.1 U/ml aprotinin, 15 μg/ml benzamidine, 10 μg/ml trypsin inhibitor) or by suspending the pellet in RIPA buffer followed by shearing the DNA 10 times through a 23 gauge needle. After centrifugation, the insoluble pellet was discarded. Protein contents were determined with the Bio-Rad DC system. Protein analysis showed that 35% of the cellular protein recovered was in the nuclear fraction and 65% was in the cytoplasmic fraction.

Immunoprecipitation, in vitro *kinase assay and immunoblotting*

Immunoprecipitations and *in vitro* kinase assays were performed as described (Fang *et al*., 1996). Briefly, 50 μg of protein from the cytoplasmic and nuclear fractions were immunoprecipitated with 2–3 μg antibodies preadsorbed to protein A-Sepharose (Pharmacia) or protein G-Sepharose (Pharmacia) in 0.1% NP-40 or RIPA buffers. The antibodies used in this work were from the following sources: antisera against cyclin A, cdk4, and p57Kip2 were gifts from Wei Jiang (The Salk Institute, La Jolla, CA), Jean Wang (UCSD, La Jolla, CA) and Wade Harper (Baylor College, Houston, TX). Antisera against cdk2 (SC-163), cdk4 (SC-260), cdk7 (SC-529), cyclin E (SC-198 and SC-247), cyclin H (SC-855) and PCNA (SC-56) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cyclin D (06-137), and histone H3 (06-570), Ad2-E1A (DP11), c-Myc (OP10), and α-tubulin were purchased from Upstate Biotechnology (Lake Placid, NY), Oncogene Science (Cambridge, MA), and Amersham (Arlington Heights, IL). Antisera against p21 (C2440) and p27 (K25020) from Transduction Laboratories (Lexington, KY) were used for immunoblotting and immunofluorescence, and anti-p21 (SC-397 and anti-p27 (SC-528) from Santa Cruz Biotechnology were used for immunoprecipitations.

Immunohistochemistry

Asynchronously growing HUT12, KRIB, and MRC-5 normal fibroblasts were fixed in 3% paraformaldehyde, 50 mM phosphate buffer, 5 mM EGTA for 5 min, permeabilized in ice-cold methanol, blocked in PBS-T (PBS with 0.1% Tween-20) and incubated for 1 h with the primary antibodies, diluted 1 : 100 for cyclins A and E, histone H3 and p21 and 1 : 50 for p27 and α-tubulin. All dilutions were made in PBS-T. FITC- or rhodamine-coupled secondary antibodies (Sigma or Pierce) were used at dilutions recommended by the manufacturer. The nuclei were stained with Hoechst 33258 (Sigma) at 1 mg/ml in PBS for 10 min. The slides were then washed in PBS-T for 15 min and embedded in Moviol (Calbiochem) containing 2.5% 1,4-diazobicyclo-[2.2.2.]-octane (DABCO) as an antifade agent.

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