Regulation of p27\textsuperscript{KIP1} in Epstein–Barr virus-immortalized lymphoblastoid cell lines involves non-apoptotic caspase cleavage

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The cyclin-dependent kinase inhibitor p27\textsuperscript{KIP1} plays a key role in controlling cell proliferation. Here we show that p27\textsuperscript{KIP1} is commonly down-regulated in B-cells immortalized by Epstein–Barr virus (EBV) (lymphoblastoid cell lines, LCLs). The significance of this event for the immortal phenotype of LCLs is implied by a requirement for active cdk2-containing complexes for continued proliferation, and by the ability of the residual p27\textsuperscript{KIP1} to associate with cdk2. The mechanism of p27\textsuperscript{KIP1} attenuation is post-translational, but inhibitor studies reveal that the mechanism does not rely heavily on the proteasome. Instead we find that LCLs contain an activity that cleaves a caspase recognition site present in p27\textsuperscript{KIP1} (DPSD\textsuperscript{139}). The activity is not associated with apoptosis and closely resembles a proliferation-associated caspase activity we previously described in the EBV-negative B-lymphoma-derived cell line BJAB. Importantly, proliferating LCLs contain a p27\textsuperscript{KIP1} product that is consistent with cleavage at this site. Inhibition of caspase(s) in vivo modulates p27\textsuperscript{KIP1} expression and strongly inhibits proliferation of IB4 cells. This inhibitor profile is identical to that displayed by the DPSD-directed caspase present in BJAB cells, suggesting that the caspase may fulfil a general role in controlling p27\textsuperscript{KIP1} expression in immortal lymphoid cell lines. Thus, apoptosis-independent cleavage appears to contribute to the maintenance of the low basal levels of p27\textsuperscript{KIP1} in B-cells immortalized by EBV.

Introduction

Epstein–Barr virus (EBV) is associated with the development of a number of human diseases including infectious mononucleosis, Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s disease, lymphoproliferative diseases, T-cell lymphomas, gastric carcinomas, post-transplant- and AIDS-associated lymphomas (reviewed in Rickinson & Keiff, 1996). EBV is able to infect quiescent (G\textsubscript{0}) primary B-cells, overriding the controls that normally limit their proliferation and resulting in the continual proliferation and outgrowth of immortal lymphoblastoid cell lines (LCLs) (reviewed in Sinclair et al., 1998). In part this is achieved by activation of the expression of cyclins and cyclin-dependent kinases responsible for the phosphorylation of pRb (Allday et al., 1995; Cannell et al., 1996; Hollyoake et al., 1995; Kempkes, 1995; Pokrovskaja et al., 1999; Sinclair et al., 1994, 1998; Spender et al., 1999; Szekely, 1995). In addition a dramatic decrease in the abundance of an inhibitor of these cdks, p27\textsuperscript{KIP1}, has been observed (Cannell et al., 1996; Spender et al., 1999).

The high abundance of p27\textsuperscript{KIP1} protein observed in quiescent primary B-cells is a feature common to many non-proliferating cells in vivo (Fero et al., 1996; Fredersdorf et al., 1997; Kiyokawa et al., 1996; Nakayama et al., 1996). In addition, in many in vitro cell culture systems the level of p27\textsuperscript{KIP1} protein is inversely regulated with the proliferation state (Bartek, 1996; Eblen et al., 1995; Firpo et al., 1994; Hengst et al., 1994; Herwig, 1997; Kwon et al., 1996; Nourse et al., 1994; Polyak et al., 1994; Ravitz et al., 1995; Reynisdottir et al., 1995; Slingerland, 1994).

Regulation of p27\textsuperscript{KIP1} is achieved through several complementary routes involving transcriptional control (Kawamata et al., 1998; Kwon et al., 1996, 1997), altered translation (Agrawal et al., 1996; Hengst & Reed, 1996; Millard et al., 1997), sequestration (Barnouin et al., 1999; Polyak et al., 1994; Poon et al., 1995; Reynisdottir et al., 1995; Sherr & Roberts, 1999; Soos et al., 1996; Vlach et al., 1996) and ubiquitin-dependent degradation by the proteasome (Pagano et al., 1995).
A further layer of regulation of p27\textsuperscript{KIP1} expression involving cleavage by caspases has recently been described in various cell models including endothelial cells undergoing growth factor deprivation-induced apoptosis (Levkau et al., 1998), apoptosis induced by a variety of agents in myeloid leukaemia cells (Eymin et al., 1999), growth-arrested hybridoma and myeloma cell lines (Loubat et al., 1999) and proliferating BJAB B-lymphoblastoid cells (Frost et al., 2001). In addition, we have shown that p27\textsuperscript{KIP1} is down-regulated by both caspase-dependent and -independent pathways in lymphoid cells undergoing apoptosis (Frost & Sinclair, 2000). p27\textsuperscript{KIP1} contains two potential caspase recognition sites, DPSD\textsuperscript{139} and ESQD\textsuperscript{180} (Eymin et al., 1999). Cleavage at these sites results in C-terminally truncated p27\textsuperscript{KIP1} proteins of 23 kDa and 15 kDa respectively (Eymin et al., 1999); the 23 kDa product (p23) is more frequently observed (Eymin et al., 1999; Frost et al., 2001; Levkau et al., 1998; Loubat et al., 1999). At least two different, as yet unidentified, caspases with differing sensitivity to z-VAD-fmk have the potential to generate p23 (Eymin et al., 1999; Frost et al., 2001; Levkau et al., 1998; Loubat et al., 1999).

In this report we extend the role of caspase or caspase-like enzymes to the regulation of p27\textsuperscript{KIP1} protein abundance in cells immortalized by EBV. We confirm that p27\textsuperscript{KIP1} is significantly down-regulated following immortalization of primary B-lymphocytes by EBV and that the resulting LCLs contain a low basal level of p27\textsuperscript{KIP1} protein; this is not present in sufficient abundance to abrogate cdk2 activity. Comparison with uninfected primary B-lymphocytes demonstrates that the regulation is achieved at a post-translational level. Ubiquitin-mediated degradation may contribute to the regulation but a more significant role is played by caspase cleavage. A caspase activity that recognizes DPSD\textsuperscript{139} of p27\textsuperscript{KIP1} can be readily detected in EBV-immortalized LCLs, in the absence of apoptosis, and this activity correlates with the presence of a 23 kDa truncated form of p27\textsuperscript{KIP1} in LCLs. Interestingly, the caspase activity is similar to one previously characterized in an EBV-negative B-lymphoma-derived cell line (BJAB) (Frost et al., 2001), which indicates that cleavage by a proliferation-associated caspase may represent a general mechanism by which p27\textsuperscript{KIP1} expression is controlled in lymphoid cell lines.

**Methods**

- **Cell culture.** The B-lymphoid cell lines were maintained in RPMI supplemented with 10% (v/v) FCS, penicillin and streptomycin. Unless otherwise indicated, cell densities were maintained at between 2 × 10\textsuperscript{5} and 8 × 10\textsuperscript{6} cells/ml to ensure that they were in continual proliferation. In some experiments, cells were seeded at 2 × 10\textsuperscript{5} cells/ml and then cultured for 5 days without re-feeding, by which time the cultures were saturated and the cells were no longer proliferating. Reactivation of the cell cultures was achieved by dilution to 2 × 10\textsuperscript{5} cells/ml in fresh medium. IB4 (King, 1980), LCL\#3 (Sinclair et al., 1994), LCL\#6 and LCL\#7 were established by immortalization of human primary B-cells with the B95-8 strain of EBV. LCL\#6 and LCL\#7 are recently established lines and had been in culture for less than 2 months at the time of these experiments. All of the cell lines are of human origin. Quiescent primary B-cells were isolated from donated human blood using affinity purification with anti-CD19-coated paramagnetic beads (Dynal) as described previously (Sinclair & Farrell, 1995; Sinclair et al., 1994).

- **Western blotting.** Total protein extracts were prepared from exponentially growing cell lines and from quiescent primary B-cells as described previously (Cannell et al., 1996). Unless otherwise indicated, the protein concentrations were normalized after quantifying the absorption at 280 nm and 100 μg of each extract was fractionated on SDS–polyacrylamide gel, transferred to PVDF membrane (Immobilon-P) and processed as previously described (Cannell et al., 1996). An additional gel was used to assess the equality of the protein concentrations by Coomassie blue staining. The p27\textsuperscript{KIP1} polyclonal antibodies C19 and N20, which are specific for the C and the N termini of p27\textsuperscript{KIP1} respectively, were purchased from Santa Cruz. The p53 monoclonal antibody DO1 was as described by Vojtesek (1992). Signals from the primary antibodies were amplified using species-specific antiserum conjugated to either horseradish peroxidase (Amersham) or alkaline phosphatase (Sigma) and detected using either ECL or ECF (Amersham). Signals detected by ECL were quantified after autoradiography using Imagemaster software (Pharmacia). Signals detected by ECF were quantified directly using the Storm phosphorimager and ImageQuantNT software (Molecular Dynamics).

- **cdk2 inhibition.** Roscovitine (Calbiochem) was solubilized in DMSO. Exponentially growing cells were cultured in the presence of roscovitine (1–50 μM) or DMSO for 8 h. The rate of DNA synthesis was determined by measurement of thymidine incorporation during a terminal 4 h pulse in medium containing [\textsuperscript{3}H]thymidine (5–50 μCi/ml; 185 kBq/ml). The cells were harvested onto paper using a semi-automatic cell harvester (Skatron) and the amount of thymidine incorporated into DNA was determined by liquid scintillation counting. Assays were carried out in triplicate and standard deviations are shown.

- **Immunoprecipitation.** NP40 extracts were prepared from exponentially growing cells as previously described (Cannell et al., 1996, 1998). Briefly, cells were washed in PBS and lysed in NP40 lysis buffer (50 mM HEPES; 1%, v/v, NP40; 0.1%, v/v, Tween 20; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 1 mM NaF; 10%, v/v, glycerol; pH 8.0). After removal of debris by centrifugation the extract was pre-cleared with protein A–Sepharose. Cleared extract derived from 4.3 × 10\textsuperscript{7} cells was used in each immunoprecipitation with either 4 μg of non-specific rabbit immunoglobulin, anti-p27\textsuperscript{KIP1} (Santa Cruz, C19) or anti-cdk2 (Santa Cruz, M2). Mock immunoprecipitations performed in the absence of extract were performed for each antibody. Antibody–protein complexes were collected with protein A–Sepharose, and washed extensively with NP40 lysis buffer. After a final wash with 50 mM Tris–HCl, pH 7.5, proteins were eluted by boiling in SDS sample buffer lacking reducing agents (4%, w/v, SDS; 20%, v/v, glycerol; 100 μg/ml bromophenol blue; 0.12 M Tris–HCl, pH 6.8). The amount of p27\textsuperscript{KIP1} and cdk2 in the immunoprecipitated proteins derived from 1 × 10\textsuperscript{6} cells was detected by Western blotting using either anti-p27\textsuperscript{KIP1} (Santa Cruz, C19) or anti-cdk2 (Santa Cruz, M2) as the primary antibody, followed by ECL.

- **Protein half-life determination.** Cells were incubated at a density of 1 × 10\textsuperscript{5} cells/ml in fresh medium for 1 h. Anisomycin was added to a final concentration of 100 μM. Cells were harvested over an 8 h period and total protein extracts were prepared, fractionated on an SDS–polyacrylamide gel and analysed by Western blotting.

- **Proteasome inhibition.** Cells were resuspended in fresh medium at a density of 2.5 × 10\textsuperscript{5} cells/ml and treated with proteasome inhibitors for a total of 320 min. The proteasome inhibitors lactacystin and...
carbobenxoxy-t-leucyl-leucyl-leucinal (MG-132) were obtained from Calbiochem and used at final concentrations of 100 µM and 10 µg/ml respectively. Calpain inhibitor II, t-acetyl-leucyl-leucyl-methionyl (LLM), was obtained from Sigma and was used at a final concentration of 10 µg/ml.

**Apoptosis assays**. The Roche Annexin-V-FLUOS staining kit was used to detect cell surface expression of phosphatidylserine as an early marker of apoptosis. The amount of cell surface-associated annexin V–fluorescein in a population of live cells was quantified using a FACS Calibur (Becton Dickinson) according to instructions supplied with the staining kit. In parallel, exponentially growing cells were treated with an agonistic Fas antibody as previously described (Frost & Sinclair, 2000) to generate a positive control for apoptosis. In addition, exponentially growing and Fas-ligated cells were deposited onto glass slides, fixed with paraformaldehyde (4%, v/v, in PBS), and stained with the DNA-specific dye Hoechst 33342 (0.1 mg/ml). The cells were inspected for nuclear degeneration by fluorescence microscopy and photographed.

**Caspace assays**. Extracts were prepared and fluorogenic caspase assays were carried out as previously described (Frost et al., 2001). Briefly, cells were washed with PBS and lysed in buffer containing 130 mM NaCl, 1% (v/v) Triton X-100, 10 mM NaPPI, 10 mM Tris–HCl, 1 mM EDTA, 1 mM PMSF, 0.25 µg/ml Pepstatin A, 10 mM NaH2PO4/NaHPO4, pH 7.5. Debris was removed by centrifugation and the cell extract collected. The ability of the resultant cell extract to cleave the three tetrapeptide–AMC substrates Ac-IETD-AMC, Ac-DPSP-AMC and Ac-ESQD-AMC was monitored as previously described (Frost et al., 2001). Briefly, 50 µl of the resultant extract was mixed with 200 µl reaction buffer (10%, v/v, glycerol; 2 mM DTT; 1 mM EDTA; 1 mM PMSF; 0.25 µg/ml Pepstatin A; 200 mM HEPES, pH 7.5; 25 µg/ml tetrapeptide–AMC substrate) in a 96-well plate. The caspase inhibitors Ac-IETD-CHO (2 µg/ml), iodoacetamide (50 mM) and z-VAD-fmk (3 µM) were also added where indicated. Reactions were allowed to proceed for 2 h at 37 °C before the AMC liberated from the tetrapeptide–AMC substrates was measured using a SpectraMax Gemini spectrofluorometer (Molecular Devices); excitation wavelength 380 nm, emission wavelength 440 nm. Background measurements generated from the reaction completed in the absence of substrate and extract were assessed and subtracted from experimental values.

**Caspace inhibition in cultured cells**. z-VAD-fmk (Calbiochem) and z-IETD-fmk (BioVision) were solubilized in DMSO and added to exponentially growing IB4 cells at the concentrations indicated. Alternatively, cultures were allowed to saturate over a 5 day period and then stimulated to re-enter the cell cycle by dilution to 2 × 10^5 cells/ml in the presence of either 2 µM z-IETD-fmk or DMSO.

**Results**

p27^KIP1 is consistently and significantly down-regulated following EBV immortalization of human B-cells

Down-regulation in the abundance of p27^KIP1 protein is a notable change following infection of quiescent primary B-cells by EBV (Cannell et al., 1996; Sinclair et al., 1998; Spender et al., 1999). We sought to ask whether this is a reproducible feature of EBV-immortalized B-cell lines and compared the expression of p27^KIP1 protein in four EBV-immortalized LCLs, IB4, LCL#3, LCL#6 and LCL#7. The latter two cell lines were recently established and had been in continuous culture for less than 2 months at the time of these experiments. As can be seen in Fig. 1(A), the four LCLs all express equivalent levels of full-length p27^KIP1 protein (with a variation of only twofold). These all fall below the level of p27^KIP1 protein observed in primary B-cells. It therefore appears that the down-regulation of p27^KIP1 protein is a consistent feature of EBV-immortalized LCLs.

**p27^KIP1 associates with cdk2 in EBV-immortalized B-cells**

We asked whether the p27^KIP1 present in LCLs was functional and able to form complexes with cdk2. An NP40 cell extract was prepared from proliferating IB4 cells and used in co-immunoprecipitation experiments to assess whether or not...
any complexes containing both p27KIP1 and cdk2 were present in these cells (Fig. 1B). These experiments revealed that p27KIP1 could be detected in cdk2 immunoprecipitates and vice versa. In addition, the relative strengths of the signals suggest that the amount of cdk2 and p27KIP1 that form a complex represents only a small proportion of the total amount of each protein present in the extract. This suggests that cdk2 is not fully inhibited by p27KIP1 in LCLs. This is supported by the ability of cdk2 isolated from LCLs to direct the phosphorylation of histone H1 (Cannell et al., 1996, 1998). Further investigation revealed that purified p27KIP1 from quiescent primary B-cells, from LCLs or expressed in vitro, is able to inhibit the kinase activity of purified cdk2-containing complexes (data not shown). Thus, in EBV-immortalized cells the low abundance of p27KIP1 may allow active cyclin–cdk2 complexes to form.

cdk2 is required for the continued proliferation of EBV-immortalized B-cell lines

We sought to question whether the activity of cdk2 complexes is required for the continued proliferation of LCLs. A specific inhibitor of cyclin-dependent kinases, roscovitine (Meijer, 1996), was applied to an EBV-immortalized cell line, IB4. Inhibition of DNA synthesis was clearly observed 8 h after the addition of 25 μM and 50 μM roscovitine (Fig. 1C). Roscovitine has been shown to selectively inhibit cdc2, cdk2 and cdk5 in vitro (Meijer, 1997). Since the kinase activity of cdk5 is restricted to neuronal cells (Tsai, 1993), we conclude that the experiment demonstrates a dependence of LCLs on cdc2 and/or cdk2. The involvement of these kinases can be distinguished by analyzing the cell cycle profile of the arrested cells; inhibition of cdk2 arrests cells in G1 with a 2n DNA content whereas inhibition of cdc2 arrests cells in G2 with a 4n DNA content. Since 60–2% of IB4 cells treated with roscovitine are arrested in G1 (data not shown), a role for cdk2 for the continual proliferation of the cells is clear. This underlines the relevance of the EBV-associated down-regulation of p27KIP1 to the continual proliferation of LCLs.

The half-life of p27KIP1 is reduced following immortalization of primary B-lymphocytes with EBV

Bearing in mind the proposed relevance of p27KIP1 down-regulation to the proliferation of EBV-immortalized cells, we were interested to elucidate the mechanism(s) by which the down-regulation is achieved. This was first approached by examining the level of mRNA encoding p27KIP1 in quiescent primary B-cells and LCLs (IB4 and LCL#3). No significant differences in the amount of p27KIP1 mRNA were detected (data not shown), suggesting that the mechanism responsible for reduced expression of p27KIP1 in the LCLs was post-transcriptional.

The half-life of p27KIP1 protein was assessed in quiescent primary B-cells and LCLs (IB4). Novel protein synthesis was halted by the addition of anisomycin and the steady state level of the pre-existing p27KIP1 protein within the cell was determined over the following 8 h. During this time period the treated cells remained viable, as judged by trypan blue exclusion assays. p27KIP1 protein appears to be very stable in quiescent primary B-cells and there was little diminution in the level observed over the 8 h of the experiments (Fig. 2, top). In contrast p27KIP1 protein in IB4 cells was turned over with a half-life of 3.2 ± 1.5 h (Fig. 2, bottom) and a similar half-life of 3.0 ± 0.7 h was obtained for p27KIP1 protein in LCL#3 cells (data not shown). The half-life of p27KIP1 protein determined in LCLs is similar to that observed in other types of proliferating cells (Hengst & Reed, 1996).

Proteasomal degradation does not play a major role in the regulation of p27KIP1 in EBV-immortalized B-cells

From the previous experiment it is clear that the p27KIP1 protein present in the primary B-cells is far more stable than that present in the EBV-immortalized cells. This suggests that one of the effects of EBV-mediated immortalization is to alter the stability of the pool of p27KIP1 protein in the primary B-cells and to further prevent p27KIP1 protein from accumulating in the LCLs.

The ubiquitin-mediated proteasomal degradation pathway has been implicated in the regulation of p27KIP1 protein in a number of cell systems (Alessandrini et al., 1997; Pagano et al., 1995); ubiquitin-conjugated forms of p27KIP1 can be found within cells and the addition of cell-permeable proteasome inhibitors results in an accumulation of p27KIP1 protein within 90 min (Pagano et al., 1995). In order to question whether the degradation of p27KIP1 that we observe in LCLs is mediated by the proteasome, we asked whether inhibiting proteasome function influences the steady state levels of p27KIP1 protein in IB4 cells. Neither the proteasome-specific inhibitor lactacystin
We previously demonstrated that apoptosis in these cells (Drexler, 1997; M. Hayes & A. J. Sinclair, unpublished data). We previously described an additional form of post-translational regulation of p27KIP1 in the EBV-negative B-cell line BJAB (Frost et al., 2001). These cells undergo dramatic cell cycle fluctuations in p27KIP1 abundance that are linked to fluctuations in an apoptosis-independent caspase activity. The caspase cleaves a fluorogenic tetrapeptide substrate (Frost et al., 2001) based on a caspase site (DPSD) previously identified in p27KIP1 (Levkau et al., 1998; Eymin, 1999). Furthermore, proliferating, non-apoptotic BJAB cells contain a truncated form of p27KIP1 consistent with cleavage at D339 (Frost et al., 2001). We asked whether a similar mechanism operates in LCLs and thus contributes to the maintenance of low basal levels of p27KIP1 in EBV-immortalized B-cells.

Western blotting of extracts from proliferating IB4 cells with an antibody directed at the N terminus of p27KIP1 demonstrates that IB4 cells, like BJAB cells, contain a cleaved form of p27KIP1 (Fig. 4A). Note that this species would not have been detected in the previous figures. The migration of the protein was consistent with truncation at D339 (data not shown). The presence of a putative caspase cleavage product in proliferating IB4 cells led us to examine whether a significant proportion of the cells were undergoing apoptosis. As a positive control for apoptosis IB4 cells were treated with an agonistic Fas antibody for 2 h and both early and late signs of apoptosis were monitored. Fig. 4(B) shows a dramatically different profile of annexin V-binding in populations of proliferating or Fas-ligated IB4 cells. Whereas the majority of the proliferating cell population (85%) shows low affinity for annexin V, and hence does not express significant amounts of cell surface phosphatidylserine, an early marker for apoptosis, the Fas-ligated cells show a large rightwards shift in annexin V-binding, indicative of the onset of apoptosis in the majority (80%) of the population. Staining with the DNA-specific dye Hoechst 33342 confirmed these differences (Fig. 4C, D). As expected, many cells in the Fas-ligated population had entered the late stages of apoptosis, with hallmark ‘popcorn-like’ nuclear morphology (Fig. 4D). However, we failed to detect any late apoptotic cells in the proliferating population (Fig. 4C) despite the presence of a small proportion of annexin V-binding cells in the population (Fig. 4B). Thus, similar to proliferating EBV-negative BJAB cells, EBV-immortalized IB4 cells contain a putative caspase cleavage product of p27KIP1, and this appears to arise independently of apoptosis.

Proliferating IB4 cells contain a z-VAD-fmk-insensitive activity that efficiently cleaves a tetrapeptide substrate based on a caspase site found in p27KIP1 (DPSD)

The caspase activity previously described in proliferating BJAB cells (Frost et al., 2001) is able to cleave tetrapeptide substrates based on one of two potential caspase sites in p27KIP1 (DPSD and the caspase-8 recognition motif (IETD). However, a tetrapeptide substrate based on a second potential caspase site in p27KIP1 (ESQD) is resistant to cleavage by the caspase. We therefore asked whether IB4 cells contained an activity with similar specificities for tetrapeptide substrates. Extracts from IB4 cells were prepared in the presence of general protease and calpain inhibitors MG-132 (LLL) or calpain II inhibitor LLM, as well as the proteasome inhibitor MG-132 (LLL) and the p53 signals were detected by ECF (bottom). The position of the molecular mass markers is shown (in kDa).

**Fig. 3.** Proteasomal degradation does not play a major role in the regulation of p27KIP1 in EBV-immortalized B-cells. IB4 cells were cultured with either the specified inhibitors or a DMSO control for 320 min, after which total protein extracts were prepared. Samples from 2.5 x 10⁶ cells were fractionated on a 10% SDS–polyacrylamide gel and the relative amount of p27KIP1 in the samples was determined by Western blotting with anti-p27KIP1 antibody (C19; top), followed by detection with ECF and quantification with ImageQuantNT software (Molecular Dynamics). Subsequently, the Western blot was re-probed with an anti-p53 antibody (DO1) and the p53 signals were detected by ECF (bottom). The position of the molecular mass markers is shown (in kDa).

nor the proteasome and calpain inhibitor MG-132 (LLL) induced an increase in the level of p27KIP1 protein in LCLs during the period of the experiment (Fig. 3), although the level of another protein that is known to be regulated by this pathway, p53, was clearly increased by three- to eightfold during this time-frame. In addition, the calpain II inhibitor LLM failed to increase the level of p27KIP1 protein in LCLs, although it was able to elevate p53 levels within the same cells, as has been described previously (Kubbutat, 1997) (Fig. 3). The use of proteasome inhibitors in many transformed cells, including LCLs is, complicated by their tendency to promote apoptosis in these cells (Drexler, 1997; M. Hayes & A. J. Sinclair, unpublished data). We previously demonstrated that p27KIP1 is down-regulated during apoptosis of lymphoid cells, including LCLs (Frost & Sinclair, 2000). It is therefore possible that proteasome inhibitors may inhibit ubiquitin-mediated degradation of p27KIP1, whilst simultaneously promoting apoptosis, resulting in the down-regulation of p27KIP1. To address this, we closely monitored the viability of the cells throughout the incubation period. From the data presented here it appears that the proteasome degradation pathway does not play a major role in the regulation of p27KIP1 protein in LCLs.

**Apoptosis-independent caspase cleavage contributes to the regulation of p27KIP1 in EBV-immortalized B-cells**

We have previously described an additional form of post-translational regulation of p27KIP1 in the EBV-negative B-cell line BJAB (Frost et al., 2001). These cells undergo dramatic cell cycle fluctuations in p27KIP1 abundance that are linked to fluctuations in an apoptosis-independent caspase activity. The caspase cleaves a fluorogenic tetrapeptide substrate (Frost et al., 2001) based on a caspase site (DPSD) previously identified in p27KIP1 (Levkau et al., 1998; Eymin, 1999). Furthermore, proliferating, non-apoptotic BJAB cells contain a truncated form of p27KIP1 consistent with cleavage at D339 (Frost et al., 2001). We asked whether a similar mechanism operates in LCLs and thus contributes to the maintenance of low basal levels of p27KIP1 in EBV-immortalized B-cells.

Proliferating IB4 cells contain a z-VAD-fmk-insensitive activity that efficiently cleaves a tetrapeptide substrate based on a caspase site found in p27KIP1 (DPSD)
Fig. 4. Apoptosis-independent caspase cleavage contributes to the regulation of p27KIP1 in EBV-immortalized B-cells. (A) Extract from exponentially growing IB4 cells was fractionated on a 15% SDS–polyacrylamide gel and p27KIP1 detected by Western blotting with an antibody directed against the N terminus of p27KIP1 (Santa Cruz, N20) and ECL. (B) Exponentially growing (black line) and Fas-ligated (grey fill) IB4 cells were stained with annexin V–fluorescein and subjected to FACS analysis to detect cell surface phosphatidylserine as an early marker for apoptosis. (C, D) Exponentially growing (C) and Fas-ligated (D) IB4 cells were deposited on glass slides, fixed and stained with the DNA-specific dye Hoechst 33342. Apoptosis-related degeneration of Hoechst-stained nuclei was detected by fluorescence microscopy and photography. Arrows indicate two nuclei displaying condensed chromatin and ‘popcorn’ morphology, characteristic of apoptosis.

Fig. 5. Extracts from proliferating IB4 cells contain a z-VAD-fmk-insensitive activity that efficiently cleaves a tetrapeptide substrate based on the caspase site found in p27KIP1 (DPSSD139). (A) Extract derived from exponentially growing IB4 cells was monitored for its ability to cleave the fluorogenic tetrapeptide substrates Ac-IETD-AMC (caspase-8 recognition motif), Ac-DPSD-AMC and Ac-ESQD-AMC (caspase recognition sites found in p27KIP1). Relative fluorescent units (RFU) were blank-corrected by subtraction of background measurements generated from reactions completed in the absence of either extract or substrate. (B) Extract derived from exponentially growing IB4 cells was monitored for its ability to cleave the fluorogenic tetrapeptide substrate Ac-DPSD-AMC in the presence of the caspase inhibitors z-VAD-fmk, Ac-IETD-CHO and iodoacetamide. RFU values presented were calculated as described in (A). (C) Extract derived from Fas-ligated IB4 cells was monitored for its ability to cleave the caspase-8 substrate Ac-IETD-AMC in the absence or presence of the caspase inhibitor z-VAD-fmk. RFU values presented were calculated as described in (A). Results presented are representative of several independent observations.
protease inhibitors and assayed for their ability to cleave a panel of fluorogenic substrates (Fig. 5 A). Ac-IETD-AMC and Ac-DPSD-AMC were efficiently cleaved by the extracts but Ac-ESQD-AMC was cleaved with a much lower efficiency.

Further similarities to the activity described in BJAB cells (Frost et al., 2001) were revealed by the use of the caspase inhibitors z-VAD-fmk, Ac-IETD-CHO and iodoacetamide. Whereas the general inhibitor of cysteine proteases, iodoacetamide, and the selective caspase inhibitor, Ac-IETD-CHO, both inhibited the DPSD-directed activity derived from proliferating IB4 cells (67% and 75% inhibition respectively), the activity was unaffected by z-VAD-fmk (Fig. 5 B). The efficacy of z-VAD-fmk was confirmed in an IETD-cleavage assay using extracts from Fas-ligated IB4 cells (Fig. 5 C). z-VAD-fmk is an inhibitor of many, but not all, known caspases; the inhibitory constants of z-VAD-fmk for capases 1–9 range from 290–2800 M

In order to produce conditions where p27^KIP1 is subject to large changes in expression, IB4 cultures were allowed to saturate over a 6 day period. This produced a population of cells with readily detectable expression of full-length p27^KIP1 protein (Fig. 7). Re-feeding of the cells resulted in a strong down-regulation of the abundance of full-length p27^KIP1. In contrast, when the cells were fed in the presence of 2 µM z-IETD-fmk p27^KIP1 expression was only partially decreased. These observations suggest that the activity of a p27^KIP1-directed caspase contributes to the proliferation of EBV-immortalized cells.

Selective inhibition of caspase(s) in EBV-immortalized cells results in a dramatic inhibition of proliferation

The importance of caspase activity(s) for the continued proliferation of EBV-immortalized cells was investigated using the cell-permeable caspase inhibitors z-VAD-fmk and z-IETD-fmk. Exponentially growing IB4 cells were treated with increasing concentrations of z-VAD-fmk (●) or z-IETD-fmk (○) for a total of 24 h. DNA synthesis was estimated by measuring the incorporation of [³H]thymidine following a terminal 4 h pulse.

Selective inhibition of caspase(s) in EBV-immortalized cells results in a dramatic inhibition of proliferation

Fig. 6. Selective inhibition of caspase(s) in EBV-immortalized cells results in a dramatic inhibition of proliferation. Exponentially growing IB4 cells at 2 x 10⁷ cells/ml were treated with increasing concentrations of z-VAD-fmk (●) or z-IETD-fmk (○) for a total of 24 h. DNA synthesis was estimated by measuring the incorporation of [³H]thymidine following a terminal 4 h pulse.

Re-feeding-induced down-regulation of p27^KIP1 in LCLs is prevented when re-feeding occurs in the presence of the caspase inhibitor z-IETD-fmk

In order to produce conditions where p27^KIP1 is subject to large changes in expression, IB4 cultures were allowed to saturate over a 6 day period. This produced a population of cells with readily detectable expression of full-length p27^KIP1 protein (Fig. 7). Re-feeding of the cells resulted in a strong down-regulation of the abundance of full-length p27^KIP1. In contrast, when the cells were fed in the presence of 2 µM z-IETD-fmk p27^KIP1 expression was only partially decreased. These observations suggest that the activity of a p27^KIP1-directed caspase contributes to the proliferation of EBV-immortalized cells.

Discussion

Changes in the expression of key elements of the cell cycle control machinery occurring as a result of the infection of quiescent primary B-cells with EBV have been characterized (reviewed in Sinclair et al., 1998). In many ways these changes resemble the events that occur in response to the physiological trations of z-VAD-fmk up to 75 µM. This eliminates any z-VAD-fmk-sensitive caspase (including caspase-8) from playing a major role in the proliferation of IB4 cells. In contrast, treatment of IB4 cells with comparatively low concentrations of z-IETD-fmk (1–10 µM) produced a dramatic inhibition of DNA synthesis (Fig. 6). This resembles the inhibition of DNA synthesis previously described in exponentially growing BJAB cells treated with z-IETD-fmk (Frost et al., 2001). These observations implicate a caspase with sensitivity to z-IETD-fmk but resistance to z-VAD-fmk in regulating the proliferation of IB4 cells. As this inhibitor profile matches that of the DPSD-directed caspase activity detected in vitro, we investigated whether the expression of p27^KIP1 in IB4 cells was affected by treatment with z-IETD-fmk.

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Discussion

Changes in the expression of key elements of the cell cycle control machinery occurring as a result of the infection of quiescent primary B-cells with EBV have been characterized (reviewed in Sinclair et al., 1998). In many ways these changes resemble the events that occur in response to the physiological
activation of B-cells by the ligation of surface IgM, CD40 and the IL4 receptor. This supports the model that at least part of the strategy employed by EBV to drive the proliferation of B-cells is to exploit the normal programme of B-cell activation.

We and others have previously demonstrated that the retinoblastoma protein phosphorylation pathway is activated following infection of primary B-cells with EBV (Allday et al., 1996; Cannell et al., 1996; Hollyoake et al., 1995; Kempkes, 1995; Pokrovskaia et al., 1999; Sinclair et al., 1994, 1998; Spender et al., 1999, Szekely et al., 1995) and we suggest that this pathway is required to facilitate the transition of infected cells through the restriction point. By analogy with other cell systems, the key kinases that direct the phosphorylation of pRb are (i) cyclin D–cdk4 and/or cyclin D–cdk6 and (ii) cyclin E–cdk2. The expression of all of these components is induced following the immortalization of B-cells by EBV (Cannell et al., 1996; Hollyoake et al., 1995; Kempkes, 1995; Sinclair et al., 1994, 1998; Spender et al., 1999, Szekely et al., 1995). In this report, we show that inhibition of cdk2 kinase activity arrests LCL cells, implying that cdk2 activity and the phosphorylation of the retinoblastoma protein are required for the continued proliferation of LCLs.

We further questioned how the cdk2 activity is regulated following EBV-mediated immortalization. In part this is achieved by de novo synthesis of both cdk2 and cyclin E (Cannell et al., 1996; Hollyoake et al., 1995; Kempkes, 1995; Sinclair et al., 1994). However, the precursor quiescent primary B-cells contain a high level of the cyclin E–cdk2 inhibitor p27KIP1 (Cannell et al., 1996; Spender et al., 1999), which displays inhibitory activity against cdk2 in vitro.

The importance of p27KIP1 protein to the regulation of cell proliferation is underscored by the myriad of molecular mechanisms that have evolved to regulate the level of p27KIP1 protein within cells (see Introduction). This description of caspase-mediated regulation of p27KIP1 in EBV-immortalized B-cells, coupled with similar observations in the BJAB B-lymphoma cell line (Frost et al., 2001), suggests caspase cleavage may represent a widespread mechanism for regulating p27KIP1 expression in lymphoid cells (see below). The potential for further regulation of p27KIP1 by caspses during apoptosis remains open. It is now well established that caspses have roles in processes other than apoptosis. Caspase-1, -4, -5 and -13, which share a preference for bulky hydrophobic residues at position 4 of the tetrapeptide caspase recognition sequence (defined as group I caspases), have a role in cytokine processing. Indeed, this substrate specificity is at odds with a role for these caspses in apoptosis, as none of the polypeptides so far identified that are cleaved during apoptosis contain hydrophobic residues at position 4 (Nicholson, 1999). Even group II caspses, which have a substrate specificity consistent with many apoptosis-related substrates (DEXD), can be found activated in the absence of apoptosis, suggesting roles in other cellular processes (Alam et al., 1999; Miossec et al., 1997). The substrate specificity of p27KIP1-directed caspases (DPSD) conforms most closely to a type II caspase recognition motif. However, the presence of proline at position 2 appears to preclude many known caspases (Thornberry et al., 1997). The insensitivity of the DPSD-directed caspase we have described herein and in proliferating BJAB cells (Frost et al., 2001) to z-VAD-fmk also distinguishes the caspase from many previously described (Garcia-Calvo et al., 1998). The presence of an apparently identical DPSD-directed caspase activity in both an EBV-negative lymphoma-derived cell line (Frost et al., 2001) and in an EBV-transformed B-cell line indicates that EBV activates a normal cellular enzyme to negate the functions of p27KIP1 during EBV-mediated immortalization. At present it is unclear whether the caspase activity is restricted to transformed lymphoid cells (a similar activity is also present in proliferating Jurkat T-cells; Frost et al., 2001) or whether the caspase is active in proliferating cells of more diverse lineages; this is currently under investigation in our laboratory.

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References


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