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An acidic environment changes cyclin D1 localization and alters colony forming ability in gliomas

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Abstract

The human glioma cell lines, U87 and T98G, were evaluated for their ability to survive and form colonies in an acidic environment of pH_{ext} 6.0. In contrast to U87, which showed an 80–90% survival rate, only 40% of T98G cells survived 6 days at pH_{ext} 6.0 and lost their colony forming ability when returned to a normocidic environment. Although both U87 and T98G cells maintain an intracellular pH (pH_i) of 7.0 at pH_{ext} 6.0 and arrest mostly in G1 phase of the cell cycle, only T98G demonstrated a major loss of cyclin D1 that was prevented by the proteasome inhibitor MG132. Colony forming ability was restored by stably transfecting T98G cells with a cyclin D1-expressing plasmid. Both U87 and T98G cells demonstrated increased cytoplasmic localization of cyclin D1 during exposure at pH_{ext} 6.0. Upon prolonged (24 h) incubation at pH_{ext} 6.0, nuclear cyclin D1 was nearly absent in T98G in contrast to U87 cells. Thus, an acidic environment triggers cytoplasmic localization and proteasomal degradation of cyclin D1.

Keywords

Cyclin D1; Glioma; Acidosis; Proteasome; pH

Introduction

High-grade astrocytomas (malignant gliomas) are the most commonly occurring primary lethal brain tumor in adults with an individual's average life expectancy being <2 years from

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the time of diagnosis. The glial tumor recurrence following surgical resection, radiation, and chemotherapy frequently occurs usually several months after these conventional therapies. Regions of spontaneous tumor necrosis in the most highly malignant types of gliomas (WHO grades III–IV) represent a consistently poor prognostic feature associated with tumor recurrence [1,2]. Surviving, but non-proliferating pseudopallisading, glioma cells border these necrotic regions. Glioma cells in adjacent hypoxic-ischemic areas have been shown to proliferate in this acidotic tumor microenvironment [3]. Investigators have reported that these acidic perinecrotic zones enhance the resistance of these transformed cells to chemotherapy and radiation therapy [4,5].

Rapidly proliferative solid tumor cells exhibit extensive non-oxidative glycolysis, but the intracellular pH (pHi) of several rapidly proliferative tumor cell types has been shown to be normocidic or alkalotic up to pHi 7.6, as compared with normal cell types [6]. Altered set point of the type I sodium-proton transport protein (NHE1) and possibly increased ATP-dependent proton extrusion [7] maintain an alkaline intracellular environment optimized for non-oxidative glycolysis in this poorly vascularized tumor environment [6,8]. Furthermore, p53 inactivating mutations in transformed cell types afford anti-apoptotic protection in an acidotic tumor microenvironment [9,10].

Cyclin D1 regulates the activity of the cell cycle dependent kinase CDK4/6, and cyclin D1 levels are regulated by its subcellular localization between the nucleus and cytoplasm during cell cycle progression [11]. Cyclin D1 is phosphorylated at Thr-286 by GSK3 β glycogen synthase kinase 3 β) and also by IKK α , a subunit of the IKK complex, which regulates NF- κ B (nuclear factor κ B) activity through phosphorylation of I κ B (inhibitor κ B) [12–14]. Phosphorylated cyclin D1 is exported from the nucleus to the cytoplasm and subjected to ubiquitin-dependent proteasomal degradation that inactivates CDK4/6 activity to contribute to cell cycle exit. Increased cyclin D1 levels in high grade gliomas correspond with increased activities of metalloproteinases and tumor invasiveness [15]. Analogously, increased cyclin D1 expression in glioblastomas (WHO grade IV glioma) is associated with increased aggressiveness and with reduced patients' life expectancies [16].

In this study, we determined that an acidic extracellular environment promotes nuclear export and degradation of cyclin D1, a failure of which can result in an undesirable tumor cell survival that might lead to tumor recurrence.

Materials and methods

Reagents, cell culture and cell lines

Human glioma cell lines U87 and T98G were acquired from the American Tissue Culture Collection (ATCC) and used between passages 5 and 10. Cells were cultured in DMEM with 10% fetal bovine serum (Hyclone) and low glucose (1 mg/ml) in a 37°C humidified incubator at 5% CO₂ and 95% room air. Cells had been tested for mycoplasma. For experiments with varying pH cells were maintained in DMEM-Hepes without bicarbonate with equimolar amount of NaCl at pH_{ext} 7.4, pH_{ext} 6.6, or pH_{ext} 6.0 that was adjusted with NaOH and incubated in a 37°C incubator at 100% room air. The medium was replaced every other day. Dead cells were detected using the trypan blue (4%) exclusion assay, and the total viable cell number manually counted with a hemacytometer as described previously [17]. The following reagents were used in this study. Puromycin (Sigma, 2 mg/ml stock solution in ETOH) was a gift from Dr. John Hershey, UC Davis Department of Biochemistry and was used to select stable T98G cyclin D1 transfectants at a final concentration of 2 μ g/ml. MG132 (25 mM stock solution in DMSO) was used as a proteasome inhibitor at the concentrations as indicated in the text.

Colony formation

Cells were trypsinized and the number of total and dead cells counted manually in the presence of trypan blue. 10^3 viable cells were plated on a 100 mm dish and incubated for a minimum of 14 days. The DMEM-Hepes growth medium was changed every third day. The medium was removed and following a rinse with PBS the cells were fixed on the plate using Tissue Fixative (Streck Laboratories) for 1 h. The colonies were stained for 1 h with a solution of rhodamine B (1% v/v in ddH₂O) and Nile Blue (0.5% v/v in ddH₂O) followed by washing several times with ddH₂O. Stained colonies, which contained more than 25 cells were counted.

Immunoblot

Immunoblots were performed as previously described [18]. Briefly, 40 µg proteins were separated by 12% SDS-gel electrophoresis, blotted electrophoretically onto Millipore membranes and subjected to immunoblotting. The following antibodies were used, mouse monoclonal anti-cyclin D1 antibodies (Neomarkers, DCS-6), mouse monoclonal anti-human cyclin D1 antibodies (BD Pharmingen) and goat anti-actin antibodies (Santa Cruz Biotechnology). Secondary antibodies were anti-horse radish peroxidase (HRP) conjugated antibodies against the individual antibody species. Chemiluminescent signals generated by ECL Plus (Amersham Biosciences) reagent were detected on autoradiography film or by Amersham Storm 860 Imaging System (Molecular Dynamics). The anti-human cyclin D1 antibodies from Neomarker cross reacted to an unidentified polypeptide migrated above the cyclin D1 polypeptide that migrated (36 KDa).

Construction of cyclin D1 expression plasmid

Cyclin D1 cDNA was isolated by RT-PCR using total RNA from human keratinocyte cell line hEP 7. First strand cDNA was synthesized employing M-MLV reverse transcriptase (Promega), with random hexamer as primer, and was amplified by PCR employing PfuUltra DNA polymerase (Stratagene). The cyclin D1 PCR primers used were 5' GCGCGGATCCCATGGAACACCAGCTCCTG 3' and 5' GCGCGTCGACTCAGATGTCCACGTCCCGC 3'. Cyclin D1 cDNA was first cloned into pCR2.1-TOPO vector (Invitrogen) following addition of a single deoxyadenosine addition to the 3' ends of the cDNA utilizing a nontemplate-dependent terminal transferase activity of *Tag* polymerase. Both strands of the cDNA were sequenced. Then correct cyclin D1 cDNA was excised utilizing *Bam*HI and *Sal*I restriction sites created by the PCR primers and cloned into pBabe puro expression vector.

siRNA experiments

siRNA for human cyclin D1 (Smartpool, #CCND1) was purchased from Dharmacon RNS Technologies. 5 nmol of siRNA were dissolved in 200 µl nuclease-free ddH₂O and 50 µl siRNA buffer (100 mM KCl, 30 mM HEPES, pH 7.5 and 1 mM MgCl₂). The final siRNA stock concentration was 20 µM. U87 glioma cells were transfected with varying concentrations of siRNA using X-tremeGENE siRNA transfection reagent (Roche) and incubated in the presence of siRNA in DMEM for 48 h. The medium was then replaced with DMEM-Hepes pH_{ext} 7.4 or 6.0, and the cells incubated for an additional 48 h. Cells were trypsinized and an aliquot stained with trypan blue and manually counted. 10^3 viable cells were plated and incubated for a minimum of 14 days until colonies were visualized. Another portion of the cells was subjected to immunoblot analyses to determine the siRNA concentration that optimally reduced cyclin D1 expression in U87 glioma cells (0.14 nmol per well).

Determination of intracellular pH_i

Intracellular pH was measured in glioma cells grown on coverslips using a spectrofluorimeter with pH_i-sensitive fluorescent dye, as described in detail elsewhere [19]. Glioma cells were grown to 70–80% confluence on collagen-coated coverslips, and loaded for 30 min at 37°C with the fluorescent ratiometric dye 2',7',-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM; Molecular Probes, Eugene, OR) in bicarbonate-free HEPES-buffered Ringer (HR), containing (in mM) 125 NaCl, 5.5 KCl, 24 HEPES, 1 MgCl₂, 0.5 CaCl₂, 10 NaOH. 10 μM nigericin (Sigma, St. Louis, MO) was used in conjunction with high-K⁺ calibration solutions of known pH_e to calibrate the dye. High-K⁺ calibration solution contained (in mM): 130 KCl, 24 HEPES, 1 MgCl₂, 0.5 CaCl₂, and 10 *N*-methyl-D-glucamine-Cl. Coverslips were rinsed to remove extracellular dye, and BCECF fluorescence was measured in a flow-through, spectrofluorometric cell (Hitachi F-2000) at 37°C perfused with HR pH 7.4 (Em = 535 nm, Ex = 507 and 440 nm). Fluorescence data was processed to yield pH_i for a population of cells [20,21]. Experiments were replicated three to five times.

Flow cytometric analysis

Cells were fixed with 70% ethanol, DNA was stained with propidium iodide. The intensity of fluorescence was measured using a Becton Dickinson flow cytometer at 488 nm for excitation and at 650 nm for emission [22]. The cell cycle profile was analyzed using Modifit's Sync Wizard (Verity Software Inc.).

Immunocytochemical staining of nuclear and cytoplasmic cyclin D1

T98G and U87MG cells grown in glass chambered slides (Nunc, Rochester, NY) were rinsed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde for 30 min at 22°C. Cells were rinsed with PBS and nonspecific staining was blocked with 10% normal goat serum/0.1% v/v Triton blocking solution for 30 min at RT. Anti-cyclin D1 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, 1:100) was added to the blocking solution and humidified slides were incubated overnight at 4°C. Following PBS rinses, a goat anti-mouse secondary (Alex Fluor 488, Molecular Probes, Eugene, OR, 1:5000) was applied for 90 min at 22°C. Cells were rinsed successively in PBS and dH₂O and coverslips secured with Aqua-Mount (Lerner Labs, Pittsburgh, PA). Fluorescent cells were visualized at 20× power using Nikophot Fluorescent microscope.

Statistics

Results are expressed as mean ± standard deviation. A minimum of three determinations were obtained for each experiment. Nonparametric Kruskal–Wallis analysis of variance (ANOVA) was performed with *post hoc* significance determined for the 95% confidence levels (*) utilizing the Mann–Whitney rank sum test (Sigma Statview; Jandel Scientific).

Results

Survival and colony forming capability of glioma cells maintained at pH_{ext} 6.0

Tumor regions of mouse mammary carcinomas and in larger ulcerated tumor areas of the same tumor are reported to have pH_{ext} values ranging from 6.4 to 7.1 and from 5.8 to 6.3, respectively [23]. We used bicarbonate-free Hepes-DMEM media having pH_{ext} values varying from pH_{ext} 6.0–7.4. The U87 and T98G glioma cells were assessed for cell survival and maintenance of clonogenic capability following exposure to normal and acidotic culture conditions. U87 cells became growth arrested during 6 days of exposure to pH_{ext} 6.0, but showed no reduction in their viable cell number and resumed growth when returned to pH_{ext} 7.4. In contrast, the number of surviving T98G cells gradually decreased, and this glioma

cell line was unable to regain normal growth at pH 7.4 after 6 days (Fig. 1a). To further demonstrate the difference in their ability to regain normal growth, these glioma cells were assessed for maintenance of their colony forming ability during acidosis (Fig. 1b). Cells that were cultured in acidotic media for the various periods were harvested and replated in Hepes-DMEM, pH_{ext} 7.4. Colonies were stained and counted after 14 days. When transferred to a normocidic environment, U87 cells maintained 70–100% of their colony forming ability following a 6 days incubation in pH_{ext} 6.0 medium. By contrast, <5% of T98G cells, which had survived in pH_{ext} 6.0 medium, established colonies when incubated in Hepes-DMEM, pH_{ext} 7.4 (Fig 1b).

Intracellular pH of U87 and T98G glioma cell lines in an acidotic environment

The pHi of both U87 and T98G glioma cell lines was determined using BCECF-AM (Fig. 1c). In the Hepes-DMEM medium, pH_{ext} 7.4 their basal pHi was determined to be alkalotic (pHi 7.4) that is consistent with intracellular values reported for other high grade human malignant glioma cell lines maintained in bicarbonate-free media at pH_{ext} 7.0 [6]. When maintained in Hepes-DMEM pH_{ext} 6.0 culture medium for at least 24 h these glioma cell lines demonstrated a pHi of 6.9–7.0.

Cyclin D1 expression is altered in T98G at acidic pH

Previously, we observed a rapid decline in cyclin D1 levels in immortalized keratinocytes that was associated with their loss of colony formation following matrix-deprivation (Nishi et al. manuscript in preparation). Therefore, we analyzed cyclin D1 levels in these two glioma cell lines and normal rat astrocytes cultured in normoacidic and acidotic culture media (Fig. 2). Cyclin D1 levels in astrocytes were reduced at pH_{ext} 6.6 and decreased further at pH_{ext} 6.0 compared with controls maintained at pH_{ext} 7.4. T98G also demonstrated reductions in cyclin D1 levels similar to that of astrocytes. By contrast, U87 cells did not demonstrate obvious reduction in cyclin D1 levels at pH_{ext} 6.6, while showing a 40% reduction at pH_{ext} 6.0 compared to about 80% in astrocytes and T98G.

Augmented cyclin D1 levels in T98G increase colony formation at pH_{ext} 6 and pH_{ext} 7.4

High basal levels of cyclin D1 in U87 glioma cells in either a normoacidic or acidotic milieu, as compared with those in T98G and normal astrocytes, indicated that cyclin D1 levels might correspond with either survival or clonogenic capacity. This hypothesis suggested that increasing cyclin D1 levels in T98G glioma cells might increase their survival and/or colony forming capability following incubation in an acidic environment. Cyclin D1 was overexpressed in stably transfected T98G glioma cells. Cells were maintained in Hepes-DMEM culture media, pH_{ext} 6.0 for 48 h followed by a colony forming assay in culture medium pH_{ext} 7.4 (Fig. 3a, b). Cyclin D1-expressing T98G cells maintained at either pH_{ext} 7.4 or pH_{ext} 6.0 exhibited more than a 3-fold increase in colony forming ability, as compared with T98G cells, which carried an empty vector. However, the colony forming ability of cyclin D1 overexpressing T98G cells cultured at pH_{ext} 6.0 was significantly lower than that of overexpressing cells cultured at pH_{ext} 7.4. The survival rates of T98G cells were not affected by cyclin D1 overexpression as compared with empty vector transfected control cells.

Reduction of cyclin D1 by siRNA in U87 cells reduces colony formation

In a converse testing of the hypothesis, cyclin D1 levels were reduced in U87 cells using cyclin D1-specific siRNA. Survival and colony forming ability were determined as described above (Fig. 3c). Cyclin D1 depletion in U87 cells was demonstrated using immunoblots, which showed nearly non-detectable cyclin D1 levels as compared with transfected control cells with no siRNA present (Fig. 3d). Survivability was not affected, but

the colony-forming capability of cyclin D1-depleted U87 cells was markedly reduced when cells were maintained in Hepes-DMEM culture medium at either pH_{ext} 7.4 and 6.0.

Cell cycle arrest in acidic medium

Cyclin D1 levels are regulated in a cell cycle dependent manner. Cyclin D1 is absent in cells in G0, increased in G1 phase and decreased when cells enter into S phase [24,25]. The cell cycle distributions of cells maintained at pH_{ext} 6.0 for up to 6 days were examined using fluorescent activated cell sorting (FACS) (Fig. 4). Analyses demonstrate that normal astrocytes, U87 and T98G glioma cells arrested randomly in all cell cycle phases when they were maintained in acidotic medium pH_{ext} 6.0 with the majority of cells however found in G1 phase. The FACS data was corroborated using immunocytochemistry. The cyclins A, B and CDK1 which are expressed in S and G2/M phases of cell cycle were detected in some cells (data not shown). When shifted back to pH 7.4 medium the FACS analysis demonstrated that the majority of U87 cells in G1 phase were able to enter S phase, while this was not the fact for astrocytes or T98G cells. These results further show, that the difference in cyclin D1 expression in T98G and U87 at pH_{ext} 6.0 is not the result of differences in the distribution of cells in the cell cycle.

Cyclin D1 is degraded under acidotic conditions

Cytoplasmic cyclin D1 is degraded by the proteasome during normal cell cycle progression [26], but it is not known whether proteasomal degradation contributed to the observed reduction in cellular levels of cyclin D1 with acidosis. Cells were incubated in culture medium for 2 h at either pH_{ext} 7.4 or pH_{ext} 6.0 and treated with varying concentrations of the proteasomal inhibitor, MG132, or not treated (Fig. 5). Total cellular levels of cyclin D1 were markedly reduced in T98G and U87 cells following a 2 h incubation at pH_{ext} 6.0, which was prevented by co-treating cells with MG132. A similar, but less dramatic increase in cyclin D1 levels with co-treatment of MG132 was also observed when T98G cells were incubated for 2 hr in pH 7.4 medium. U87 glioma cells showed higher basal levels of cyclin D1 than either T98G cells. U87 levels of cyclin D1 were augmented following a 2 h incubation with MG132. The result indicates the involvement of the proteasomal pathway in regulating cellular cyclin D1 levels under acidotic conditions in both T98G and U87 cells.

Cyclin D1 is localized in the cytoplasm at pH 6.0

Cyclin D1 is exported to the cytoplasm for proteasomal degradation in S-phase during cell cycle progression. Immunocytochemical localization of cyclin D1 was performed on U87 and T98G glioma cells that were incubated in pH_{ext} 6.0 medium for 8 and 24 h and compared to cells that were maintained at pH_{ext} 7.4 (Fig. 6). T98G cells demonstrated a time-dependent reduction in nuclear cyclin D1 levels following 8 and 24 h at pH_{ext} 6.0 compared to pH_{ext} 7.4. This corresponded to a significant increase in cytoplasmic cyclin D1 in T98G. Cytoplasmic cyclin D1 was also detected in U87 cells although the level of nuclear cyclin D1 was nearly unaltered at pH_{ext} 6.0 compared to cells at pH_{ext} 7.4. This demonstrates that an acidic environment causes cytoplasmic localization of cyclin D1.

Discussion

We demonstrate here that an acidic environment enhances the nuclear export and the degradation of cyclin D1. The original function described for cyclin D1 is the formation of a complex with CDK4/6, which phosphorylates and inactivates the retinoblastoma protein pRb, triggering cell cycle progression from G0/G1 to S-phase [27]. Cyclin D1 is not required for cell cycle progression in the absence of a functional pRb, as shown in pRb $-/-$ knockout cells [28]. Both T98G and U87 cell lines contain a functional pRb and their pRb-dependent cell cycle arrest has been described [29]. The acidosis-induced cell cycle arrest

does not appear to be caused by lack of cyclin D1. T98G, which showed a severe reduction in cyclin D1 and U87, which maintained cyclin D1 levels, were both arrested randomly in the cell cycle when cultured in acidic medium. In addition phosphorylation of pRb at Ser807/811, which are phosphorylation sites for CDK4/6, was observed in U87 cultured under acidic condition (data not shown). However, preservation of cyclin D1-cdk4/6 complex in the nucleus of cell cycle arrested cells might prevent losing their capability to proliferate during acidosis. Recently cyclin D1 has been shown to have a transcriptional cofactor activity in a cdk-independent manner [30,31]. Although a clear link between this activity of cyclin D1 and human cancers remained to be established, it may contribute to colony formation as well.

During normal cell cycle progression nuclear export of cyclin D1 is triggered by its phosphorylation of Thr-286 by GSK-3 β and IKK α and coupled with its proteasomal degradation [12–14], although the exact role of both kinases pertaining to cyclin D1 degradation is not completely established. A brief 2 h exposure to pH_{ext} 6.0 medium decreases total cellular levels of cyclin D1 in T98G glioma cells and is associated with cytoplasmic mobilization. One possibility is that AKT is inactivated, which leads to activation of GSK3 and cyclin D1 phosphorylation. However, we observed that AKT was still phosphorylated at acidic pH and therefore most probably active. Another possibility is that IKK α , which also phosphorylates Thr-286 of cyclin D1 [14], is the active kinase. Free cyclin D1 (unbound to CDK4/6) was also reported to be degraded without phosphorylation of Thr-286 [32]. Mirk kinase, which has been recently reported to phosphorylate cyclin D1 at Thr-288 in G1 phase remains as another possible alternative kinase, which is responsible for this cyclin D1 degradation [33]. Mutations in N-terminal lysins of cyclin D1 impair its proteasomal degradation. Thus, pH-dependent impairment of cyclin D1 degradation could result from mutations in the naturally occurring glioma cell lines [34]. However, mutations in the structural gene of cyclin D1 in glioblastomas are very rare events [35], while overexpression of cyclin D1 is reported in more than 50% of astrocytic tumors [36].

Immunocytochemical staining demonstrate both nuclear and cytoplasmic localization of cyclin D1 in U87 glioma cells incubated in pH_{ext} 6.0 medium for 8 and 24 hr, whereas cyclin D1 in T98G was exclusively cytoplasmic. This could suggest that U87 maintains a higher synthesis rate of cyclin D1 compared to degradation. Alternatively, degradation of cyclin D1 may occur at a slower rate in this cell line as compared with T98G cells or astrocytes. In fact a decrease in the cyclin D1 levels in U87 cells was not observed at pH 6.6, unlike astrocytes and T98G cells.

Our pHi measurements demonstrate that extracellular acidosis of pH_{ext} 6.0 corresponds with an pHi of 6.9–7.0 for both glioma cell types. A pHi of 6.9–7.0 has been reported for normal astrocytes [6,37] and other normal brain cell types. Therefore the effect of the extracellular acidification observed in T98G and U87 glioma cells is not the effect of a non-physiological pHi, rather it appears to be a cell's response to their environment. Our data indicate that nuclear levels of cyclin D1 play an essential role for transformed cells to maintain their proliferative capability in an acidotic, perinecrotic tumor environment and that the population of such cells that retain nuclear cyclin D1 might serve as a source of tumor recurrence.

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Fig. 1.

Cell survival and colony formation. Cells were cultured at pH 7.4 and at 0 h shifted to Hepes-buffered medium with the indicated pH in an incubator without CO₂. **(a)** Cells were stained with Trypan blue and live cells were counted (circle: U87; square: T98G). **(b)** Colony forming ability of 1000 cells was determined (circle: U87; square: T98G). **(c)** The intracellular pH was measured in U87 (grey bars) and T98G (white bars) cells when exposed to medium with pH at 7.4 or 6.0

Fig. 2.

Cyclin D1 levels in neutral pH and acidic environment. Normal rat astrocytes, U87 and T98G glioma cells were grown until 50–70% confluence and shifted to medium with the indicated pH. Cells were incubated for 48 h and harvested for use in immunoblots. Cyclin D1 from the immunoblots was quantified by densitometry, and the relative values normalized to actin and expressed as percent (%)

Fig. 3. Alterations of cyclin D1 expression in T98G and U87 compared to colony formation. **(a)** T98G stable transfectants were cultured at pH7.4 and at 0 h shifted to Hepes-buffered medium with the indicated pH in an incubator without CO₂ for 96 h and colony-forming ability of cells was determined. **(b)** Immunoblot with anti-cyclin D1 and anti-actin antibodies from T98G cells incubated for 48 h at indicated pH. **(c)** Cyclin D1 knockout by siRNA in U87 glioma cells. Cells were transfected with 2 μ g cyclin D1 siRNA for 48 h at pH7.4. Then medium was changed to Hepes-buffered medium pH7.4 or pH 6.0 and incubated for another 48 h and colony-forming ability of cells was determined. **(d)** Immunoblot with anti-cyclin D1 and anti-actin antibodies from U87 cells incubated at indicated pH and as described in c) (* indicates statistical pairwise significance at $P < 0.05$)

Fig. 4. Fluorescent activated cell sorting (FACS) of rat astrocytic primary cultures, U87 and T98G. All cells incubated at $\text{pH}_{\text{ext}} 6.0$ show increases in G1 cells. The number of G1 cells decline when maintained in Hepes DMEM, $\text{pH}_{\text{ext}} 7.4$ for 48 h. All cells maintained in a low $\text{pH}_{\text{ext}} 6.0$ environment demonstrate cell cycle arrest in all phases of the cell cycle. (a) normal rat astrocytes, (b) U87, (c) T98G

Fig. 5.

Degradation of cyclin D1. U87 and T98G cells were incubated at pH 6.0 or 7.4 for 2 h with the proteasome inhibitor MG132 before harvesting cells for immunoblot analysis

Fig. 6.

Cytoplasmic localization of cyclin D1. U87 and T98G cells were grown on glass slides and incubated for the indicated times at pH 7.4 or 6.0 and stained as described in Material and Methods. The number of cells with cytoplasmic staining was determined by manual cell counts. Only cells lacking nuclear staining were counted as cytoplasmic staining: T98G 8 h at pH 7.4, 17% (16 of total 95); 8 h at pH 6.0, 54% (47 of total 86); 24 h at pH 6.0, 85% (76 of total 89); U87 8 h at pH 7.4, 12% (13 of total 113); 8 h at pH 6.0, 8% (16 of total 196); 24 h at pH 6.0, 25% (20 of total 80)